GLUTAMATES VOL 7 #39
ADDENDUM

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GLUTAMATES

ADDENDUM

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Amino Acids I FED PROC 31 '72

2814 NUTRITION

MONOSODIUM GLUTAMATE METABOLISM IN THE NEONATAL PIG. G. L. Baker*, L. D. Stegink*, and L. J. Filer, Jr. The University of Iowa, Iowa City, Iowa 52240.

Recent reports indicate that the arcuate nucleus of the hypothalamic region is particularly vulnerable to monosodium glutamate (MSG) induced damage in the suckling mouse or rat, but not in the dog or monkey. The toxic compound appears able to penetrate the neural system of the susceptible species during the first days of life, but is unable to do so upon maturation of the animal. The human infant at birth is a more mature organism than the suckling mouse or rat, thus we have studied the effect of MSG load on the newborn pig. MSG, dissolved in water or infant formula was administered to 3 day old pigs by stomach tube, and peripherial blood, portal blood and tissue samples were obtained with time for amino acid analyses. The levels of MSG studied (0.01, 0.1 gm/kg) were those which could have been fed a human infant ingesting all of its calories from a commercial infant food preparation containing the highest level of MSG. After administration of U-^{14}C-MSG, only glutamate, glutamine and alanine contained significant quantities of radioactivity in the amino acid fraction, and the plasma and tissue levels of these amino acids were not significantly different from those of control animals. Since the plasma glutamate levels are strikingly elevated at the dose of MSG required to produce the lesion in the suckling mouse, it seems unlikely that any neurotoxic effect would be observed at the dose levels studied. (Supported in part by a grant-in-aid from the Gerber Products Co.).

2815 NUTRITION

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 72, 428–436 (1957)

Quantitative Nutritional Studies with Water-Soluble, Chemically Defined Diets. III. Individual Amino Acids as Sources of "Non-Essential" Nitrogen

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Received April 18, 1957

Introduction

With the announcement that this might be the final report on the amino acid requirements of the rat, Rose, Oesterling, and Womack in 1948 (1) summarized the findings of the previous 18 years, and again investigated the bothersome position of glutamic acid in amino acid nutrition. They reiterated the observation that the exclusion from food of any one of the nine essential amino acids (lysine, tryptophan, histidine, phenylalanine, leucine, isoleucine, threonine, methionine, and valine) was followed by profound nutritive failure, loss in weight, and eventual death, while deletion of arginine resulted only in a lesser rate of gain of body weight. Isonitrogenous diets were prepared, and over a period of 28 days of feeding young, male weanling rats, the mean gain on a diet containing only the ten "essentials" was 79.1 ± 0.82 g., on a diet containing the ten "essentials" plus glutamic acid 91.6 ± 1.07 g., and on a diet containing all 19 amino acids, such as occur in proteins, 108.4 ± 1.28 g. Thus, the addition of glutamic acid furnished a distinct stimulant to growth, but when all of the "non-essentials" were present, still better growth occurred. From these data it was concluded that a mixture of "essentials" and "non-essentials" such as occurs in protein is quite superior in nutritive quality to one containing the ten "essentials" only: "The task of synthesizing [the non-essentials] simultaneously appears to present too great a burden upon the chemical resources of the cells to permit the latter to keep pace with the needs of the organism for optimum growth" (1).

When glutamic acid was added to the mixture of only the ten "essential" amino acids an increase in growth occurred, but when deleted from the mixture of 19 amino acids no change in the growth rate occurred (1). This confronted the investigators with a dilemma in respect to the designation of glutamic acid as "essential" or "non-essential." It was resolved by reference to the behavior of arginine, "the least effective of the essentials" (1), which when deleted from the 19 amino acid diet *does* result in a decrease in the rate of gain of body weight. Glutamic acid was therefore classed as a dispensable amino acid, although the arbitrary character of this designation was recognized. For much the same reasons, aspartic acid was also classed as dispensable. The "non-essential" amino acids included therefore, glycine, alanine, serine, cystine, tyrosine, aspartic acid, glutamic acid, proline, and hydroxyproline.

The comparison of the effects on growth elicited by the three diets mentioned above suggested that if the "non-essential" amino acids were truly dispensable, the stimulating effect which their presence had on growth might be imitated by any source of utilizable nitrogen. In a brief note in 1949 Lardy and Feldott (2) reported that the addition of diammonium citrate as a source of "non-essential" nitrogen to a diet containing the ten "essential" amino acids would produce a weight gain over and beyond that produced by the basal diet alone. Almost simultaneously, Rose and his colleagues (3) carefully established diets in which the ten "essential" amino acids were employed at their minimal level; when various forms of "non-essential" nitrogen were added to this basic diet an increase in growth rate was observed. Urea and glycine did not appear to be as efficient as the ammonium sources (3). The actual utilization, however, of urea nitrogen by the rat in the biosynthesis of "non-essential" amino acids was shown by the feeding of N^{15}-labeled urea under the above conditions (4); the cystine, glutamic acid, aspartic acid, and tyrosine isolated from the tissues were strongly labeled with N^{15}. The fact that some growth occurred on only the ten "essentials" was somewhat puzzling, and it was considered possible that the D-forms of the racemic valine, isoleucine, and threonine components which were presumed to be inert as far as growth was concerned may yet have been metabolized and furnished a source of ammonia for the synthesis of the "non-essential" amino acids of the tissues. Later experiments by Frost (5) revealed that the ability of the rat to convert "essential" amino acids to "non-essential" amino acids was not as good

as the ability to convert other sources of nitrogen to these compounds. As Hopkins had pointed out many years earlier (6), dietary nitrogen is employed for the synthesis of compounds other than what later was called the "non-essential" amino acids, and presumably the nonspecific sources such as ammonium salts are more readily suited for this purpose han are the "essential" amino acids.

Other than L-glutamic acid and glycine (3), individual amino acids of the "non-essential" variety have not been employed as sole sources of "non-essential" nitrogen. With the hope that a better understanding be reached of the extent to which each of these amino acids may be employed, and further knowledge be gained of their greater or lesser dispensability as each was converted into all of the other "non-essential" components, the present study was instituted.

Experimental

The animals, dietary components, and basal diet were the same as described in the first paper of this series (7). The amino acid complement was composed of the ten "essentials" to a level, per kilogram of diet, of 9.5 g. total N [cf. Table I of Ref. (7)]. This diet was fed in 50% aqueous solution more or less as a base line for the experiments to follow. Other diets studied were those in which single nitrogenous components were added at the expense of the glucose component, at 15.7 or 12.5 g. N levels, thus making the total N 25.2 and 22.0 g., respectively. Each of these diets as before (7) was prepared at 24–28° as a 50% solution in water (2 cal./ml.) and offered to the animals *ad libitum*. Each animal was housed in a suspended separate cage provided with a grated floor and an inverted tube containing tap water which the animals were allowed to drink *ad libitum*. Six animals or more were employed with each diet studied, and the entire experiments were checked by repetition. Dietary intake and weight changes were measured for each animal. Fecal excretion, as usual, was scanty and infrequent.

Results and Discussion

The pertinent data are given in Table I with averaged results, while the growth curves are delineated in Figs. 1–4. No completely satisfactory base line for these experiments was possible. Obviously the addition of "non-essential" nitrogen to the basal diet increased the total nitrogen of the diet, and a firm comparison with results on the basal diet alone would not be quite proper. On the other hand, the alternative of increasing the "essentials" in the basal diet to a total level of 25.2 or 22.0 g. N so as to make this diet isonitrogenous with the subsequent diets composed of "essentials" plus a single "non-essential" would not be proper, either, for the "essential" complement would be different tin the former and latter diets.

TABLE I

Average Growth Response of Six Male, Sprague-Dawley Weanling Rats per Group when Individual Sources of "Non-Essential" Nitrogen were Added to the Basal Diet [Table I of Ref. (7)]

Diet No.	Component added	Total N of component added	Time on diet	Average starting weight	Average weight gain over period	Average intake over period		Ratio of weight gain to intake
		g.	*days*	*g.*	*g.*	*ml.*	*g.*	
39	None	0	21	50	9.0	192	96	0.09
1	L-Alanine	15.7	21	47	60.5	326	163	0.37
38	L-Proline	15.7	21	50	46.7	270	135	0.35
8	L-Arginine.HCl	15.7	24	46	58.5	382	191	0.31
9	D-Arginine.HCl	15.7	20	46	27.3	254	127	0.21
36	Glycine	15.7	21	49	14.5	166	83	0.17
37	L-Hydroxyproline	15.7	21	49	−2.4	108	54	—
31	L-Butyrine	15.7	21	49	7.5	130	65	0.12
32	D-Alanine	15.7	21	49	40.2	280	140	0.28
33	L-Serine	15.7	21	48	−8.0	80	40	—
7	L-Cysteine	15.7	—	45	All died	—	—	—
29	Urea	15.7	21	49	24.8	266	133	0.18
30	Ammonium acetate	15.7	21	49	48.2	314	157	0.32
10	L-Alanine	12.5	21	45	61.2	318	159	0.38
12	Ammonium L-glutamate	12.5	21	45	62.2	330	165	0.37
13	L-Glutamine	12.5	21	45	61.3	336	168	0.37
14	Ammonium L-aspartate	12.5	21	45	62.2	342	171	0.37
15	L-Asparagine.H_2O*	12.5	21	45	(56.0)	(360)	(180)	(0.31)

* Slow precipitation of L-asparagine from the diet solution rendered results doubtful.

The total N per kilogram of diet for the first diet described in Table I was 9.5 g., while that for the next 12 diets was 25.2 g. Because of the relative insolubility of L-asparagine in the diet solutions, this compound was furnished at a lower level, and for comparison purposes the last five diets in Table I contained, per kilogram, 22.0 g. of total N. The diets providing the best growth were Nos. 1, 10, 12, 13, 14, and 38, in which, respectively, L-alanine, ammonium L-glutamate, L-glutamine, ammonium L-aspartate, and L-proline were individually furnished as the sole source of "non-essential" nitrogen. Despite the uncertainty of the results with L-asparagine, it is not improbable that this compound should belong to this effective group. The growth responses on these better diets were still appreciably less than those on No. 3 and much less than

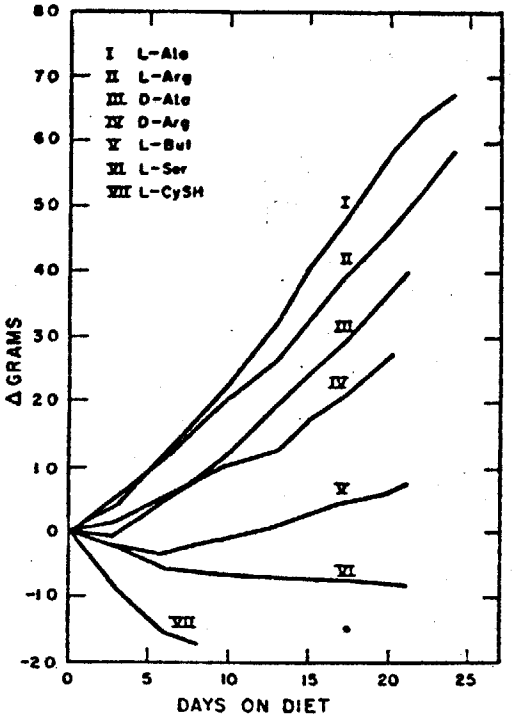

FIG. 1. Growth curves of weanling male rats when individual L-amino acids as sole source of "non-essential" nitrogen were added to the basal diet. All diets isonitrogenous.

on No. 26, described in the first paper in this series (7), which contained a larger assortment of "non-essential" amino acids, although all possessed nearly the same total nitrogen.

The addition of the D-isomers of alanine and of arginine (diets Nos. 32 and 9, respectively) to the basal diet accelerated growth, but not to the same extent as that produced by the corresponding L-isomers (diets Nos. 1 and 8, respectively). It is not improbable that these D-isomers are partially effective by virtue of their inversion to the corresponding L-isomers. Ammonium acetate was more effective than either urea or glycine, which confirms the results of Rose *et al.* (3). L-Serine, L-hydroxyproline, and L-cysteine at the levels used proved to be toxic to the animals (Table I). The striking toxic effect of L-hydroxyproline at concentrations at which L-proline stimulates growth had already been noted by Rose and his associates (8).

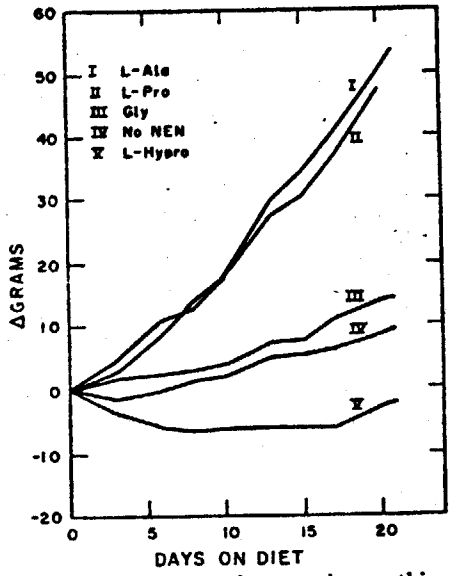

FIG. 2. Growth curves of weanling male rats when nothing was added to basal diet (curve IV) and when individual amino acids as sole source of "non-essential" nitrogen were added to the basal diet. All diets isonitrogenous.

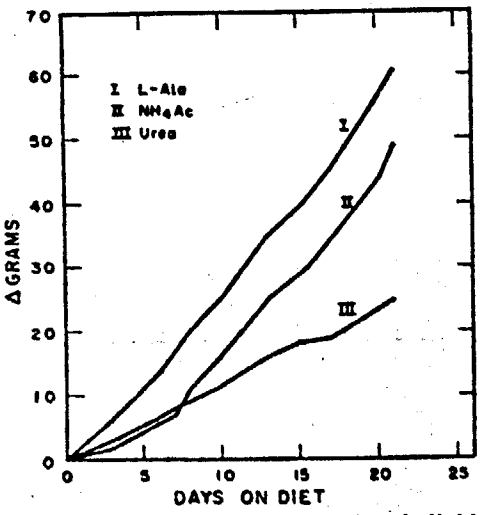

FIG. 3. Growth curves of weanling male rats when individual compounds as sole source of "non-essential" nitrogen were added to the basal diet. All diets isonitrogenous.

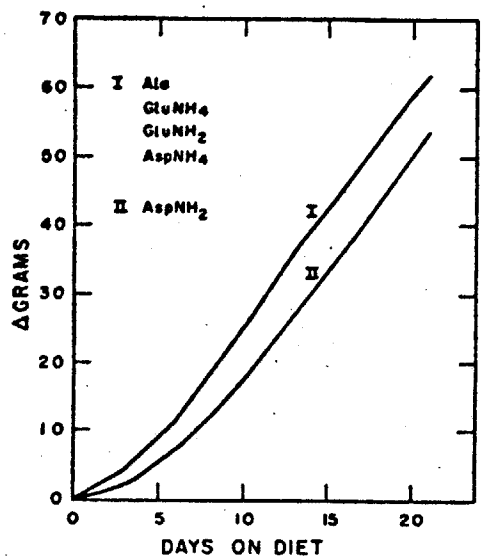

FIG. 4. Growth curves of weanling male rats when individual amino acids as sole source of "non-essential" nitrogen were added to the basal diet. All diets isonitrogenous.

Of interest are the ratios of average weight gain to the intake over the periods during which the animals were on the diet (Table I), for these ratios are obviously smaller the poorer the diet. Thus, the data in Table I can be broken down as follows:

Range of weight gains, *g.*	7.5–27.3	40.2	48.2–62.2
Range of ratios	0.09–0.21	0.28	0.31–0.38

With L-hydroxyproline on which the animals lost 2.4 g., the intake was 54 g.; and with L-serine on which the animals lost 8.0 g., the intake was 40 g., over the same period of time on the two diets. These intakes were obviously too small to support maintenance, much less growth. The lower ratio values are obviously due to an intake which is not reflected in growth, as if the animals were attempting to compensate for a failure to transform dietary material into tissue by ingesting the diet at a level higher than was needed.

On the better diets in Table I, the range of ratios was 0.31–0.38. The still better diets described in Table II of the first paper in this series (7) yielded ratios between 0.38 and 0.41, only the poorest diet in this table yielding a ratio of 0.34. None of these data are unexpected, for

there is an obvious general relation between intake and weight gain, but the quantitative precision of these data is of interest.

It would appear that, under the conditions employed, some amino acids may be converted by the rat to the full complement of tissue protein "non-essential" amino acids more rapidly than others, and at a rate sufficient to produce fair growth of the animal. These rates of conversion are a reflection of the metabolism experienced by each of the amino acids in the body of the animal. The different fate of each is no doubt the reason why these components must be present in definite ratios to each other in order to achieve a maximum growth response [cf. (7)]. No amino acid is "non-essential" for maximal growth.

SUMMARY

The basal diet described earlier, and consisting of the ten "essential" amino acids to 9.5 g. total N, B-vitamins, ascorbic acid, salts, and glucose was dissolved in water to 50% concentration and offered to weanling male rats. Over a 21-day period there was an average weight gain of 9 g. or about 0.45 g. per day per animal. Various amino acids and other nitrogenous compounds were individually added to the basal diet at the expense of an equal amount of the glucose component, and the resulting diets, also in 50% aqueous solution, were likewise offered *ad libitum* to weanling rats. These supplemented diets were all isonitrogenous, i.e., either 25.2 or 22.0 g. total N/kg.

The diets providing the best growth, about 3 g. per day, were those in which L-alanine, ammonium L-glutamate, L-glutamine, ammonium L-aspartate, and L-proline were individually furnished as the sole source of "non-essential" nitrogen. The additions of the D-isomers of alanine and of arginine to the basal diet accelerated growth but not to the same extent as that produced by the corresponding L-isomers when also employed singly. Ammonium acetate proved more effective in promoting growth than either urea or glycine, while L-serine, L-hydroxyproline, and L-cysteine, at the levels used (15.7 g. total N/kg. diet) proved to be toxic to the animals.

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NORTHBROOK, ILLINOIS 60062

REPORT TO

WM. UNDERWOOD CO.

HOST-MEDIATED ASSAY FOR DETECTION OF MUTATIONS INDUCED BY AC'CENT BRAND MONOSODIUM-L-GLUTAMATE

MAY 3, 1973

IBT NO. 632-03039

Industrial BIO-TEST *Laboratories, Inc.*
1810 FRONTAGE ROAD
NORTHBROOK, ILLINOIS 60062

May 3, 1973

Dr. A. G. Ebert, Director
Product Safety and Regulatory Affairs
Wm. Underwood Co.
One Red Devil Lane
Watertown, Massachusetts 02172

Dear Dr. Ebert:

Re: IBT No. 632-03039 - Host-Mediated Assay for Detection of Mutations induced by Ac'cent Brand Monosodium-L-Glutamate

We are submitting herewith our laboratory report dated May 3, 1973, prepared in connection with the above study.

Very truly yours,

J. C. Calandra

J. C. Calandra
President

JCC: sjn

REPORT TO

WM. UNDERWOOD CO.

HOST-MEDIATED ASSAY FOR DETECTION OF MUTATIONS INDUCED BY AC'CENT BRAND MONOSODIUM-L-GLUTAMATE

MAY 3, 1973

IBT NO. 632-03039

I. Introduction

Mutations can arise spontaneously or can be induced. Systems involving bacteria, insects, or molds have been devised to investigate compounds that may induce genetic changes. However, the relevancy of these tests, as related to mammals, is difficult to measure. The host-mediated assay is a method of screening potential mutagens in which both a bacterial system and a mammalian system are used. Male albino rats are treated with a test compound. After a period of treatment, during which time the compound can be metabolized, the host animal is inoculated with bacteria in which reverse mutations can be measured. Following exposure to the compound and/or its metabolites in vivo, the bacteria are recovered and the number of revertants (mutants) is determined.

II. Summary

The possible effects of Ac'cent Brand Monosodium-L-Glutamate (MSG), Batch #64047-1179, and its metabolites upon bacteria indirectly exposed to these compounds were investigated using the host-mediated assay. Albino rats were treated with oral doses of either 0.2 g MSG/kg of body weight (T-I) or 5.7 g MSG/kg (T-II) daily for 14 consecutive days. Twenty-four hours after the last dose, each animal was inoculated with S. typhimurium, strain G46, a histidine auxotroph. After a 3 hour residence in the peritoneal cavity, the bacteria were recovered and the number that had mutated (reverted) to their protrophic form was determined. Dimethylnitrosoamine (DMN) was administered to rats as a single intramuscular injection of 100 mg/kg to serve as a positive control.

The number of bacteria recovered from MSG treated animals was not increased over that of the control animals (spontaneous rate). DMN-treated animals had a 3 to 5 fold increase over the control rate. MSG was not mutagenic in this test system using subchronic oral doses of 0.2 or 5.7 g/kg.

Respectfully submitted,

INDUSTRIAL BIO-TEST LABORATORIES, INC.

Report prepared by: Dennis Arnold

Dennis Arnold, B.S.
Group Leader
Genetic Studies

Report approved by: Gerald L Kennedy Jr

Gerald L. Kennedy, Jr., B.S.
Section Head, Toxicology

M. L. Keplinger

M. L. Keplinger, Ph.D.
Manager, Toxicology

sjn:mcb

III. Procedure

A. Outline of Experiment

Male Charles River albino rats weighing between 250 and 300 g served as the test animals and Salmonella typhimurium, strain G46, a histidine auxotroph, was used as the bacterial mutagenic indicator. The material tested was Ac'cent Brand Monosodium-L-Glutamate (hereafter referred to as MSG), Batch No. 64047-1179. Dose levels employed were fractions of the reported $AOLD_{50}$ (19.9 g/kg); 0.2 (T-I) and 5.7 (T-II) g/kg. All doses were delivered orally (gavage) with T-I animals being treated once per day with a 20 percent solution (w/v) in distilled water and T-II animals twice per day with a 30 percent solution (w/v) in distilled water (2.85 g/kg per dose for a total 5.7 g/kg per day). Control animals were treated 2 times per day with distilled water in volumes equivalent to those received by T-II animals.

Groups of 4 animals each were treated daily for 14 days with their respective solution. After 1 week of treating animals in Trial 1, animals in Trial 2 (4 per group) were started on their dosing regimen. Dimethylnitrosoamine (DMN) was used as the positive control material.

The organization of groups is presented in Table I.

TABLE I

TEST MATERIAL: MSG

Host-Mediated Mutagenic Study - Albino Rats

Organization of Groups

Group	MSG Dose Level (g/kg)	DMN Dose Level (mg/kg)	Number of Animals Trial 1	Trial 2
C	0	0	4	4
PC	0	100	4	4
T-I	0.2	0	4	4
T-II	5.7	0	4	4

Note: In order to ensure recovery of bacteria from at least 3 animals per group, 4 animals from each group were inoculated at each trial. DMN (dimethylnitrosoamine) was administered as a single intramuscular injection at the time of inoculation.

B. Test Procedure

Animals were treated daily for 14 days with T-I animals receiving a single oral dose (gavage) of the 20% MSG/water solution and T-II animals being treated b.i.d. with the 30% test solution. Control animals were treated b.i.d. with distilled water. The study was divided into 2 separate trials. Animals were weighed daily to ensure accurate dosage.

On the day prior to each trial, sterile nutrient broth was inoculated with S. typhimurium and incubated at 37°C overnight. Five ml of the overnight culture was added to each of 2 sterile flasks containing 50 ml of nutrient broth and incubated at 37°C. After 90 minutes, 4 animals from each group were inoculated with 5 ml of the standardized culture via an intraperitoneal injection. This was done in order to ensure adequate bacterial recovery from 3 animals per group. All test animals were inoculated 24 hours following the last oral dose of MSG. At the same time positive control males received an intramuscular injection of 100 mg DMN/kg of body weight. The animals were then housed individually.

Three hours post-inoculation, the animals were sacrificed by carbon dioxide asphyxiation. Each animal was swabbed with 70 percent alcohol and was given a 1 ml intraperitoneal injection of sterile, normal saline. The peritoneal cavity was then aseptically opened and as much fluid as possible aspirated with a sterile syringe. The aspirate was transferred to a sterile tube and placed in an ice bath.

Three, serial 100-fold dilutions of the peritoneal washings were prepared in sterile, normal saline. A 0.2 ml aliquot of the diluted peritoneal fluid was added to 1.5 ml of sterile agar containing histidine*. A 0.4 ml aliquot of the undiluted aspirate was added to 1.5 ml of sterile agar without histidine. Each agar preparation was overlayed on sterile agar in petri dishes. Duplicate samples of each were made. The plates were incubated at 37°C for 48 hours.

C. Calculation of Mutation Rate

After the incubation period, colony counts on all plates were made and recorded. Typical Salmonella colonies appearing on the histidine deficient agar were counted as mutants.

The formula for the calculation of the mutation rate is:

$$\text{Reversions per } 10^8 \text{ Survivors (mutation rate)} = \frac{\text{Total revertants per ml of aspirate on deficient agar}}{\text{Total organisms per ml of aspirate on complete agar}} \times 100$$

* L-(-)-Histidine, Eastman Kodak Company, Rochester, New York.

IV. Results

No unusual reactions were noted among animals in any group and no deaths occurred.

The results of the 2 trials conducted are presented in Tables II and III. The numbers of induced reverse mutations for test animals were not above the spontaneous rate obtained. Mutation rates for animals in either test group compared favorably with those for control animals. Positive control animals had a 3 to 5 fold increase over the spontaneous rate.

TABLE II

TEST MATERIAL: MSG

Host-Mediated Mutagenic Study - Albino Rats

Normal and Revertant Bacteria Counts

Trial No. 1

Group	Animal Number	Organisms per ml of Aspirate Total x 10^6	Revertants	Mutation Rate
C	1	330.00	8.75	2.65
	2	310.00	8.75	2.82
	3	327.50	6.25	1.91
	Average			2.45
PC	1	345.00	22.50	6.52
	2	350.00	21.25	6.07
	3	432.50	46.25	10.69
	Average			7.98
T-I	1	302.50	7.50	2.48
	2	327.50	6.25	1.91
	3	315.00	5.00	1.59
	Average			1.98
T-II	1	335.00	12.50	3.73
	2	320.00	3.75	1.17
	3	327.50	2.50	0.76
	Average			1.91

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REPORT TO

WM. UNDERWOOD CO.

MUTAGENIC STUDY WITH AC'CENT BRAND MONOSODIUM-L-GLUTAMATE IN ALBINO MICE

MAY 9, 1973

IBT NO. 632-03040

Industrial **BIO-TEST** *Laboratories, Inc.*
1810 FRONTAGE ROAD
NORTHBROOK, ILLINOIS 60062

May 9, 1973

Dr. A. G. Ebert
Director, Product Safety
and Regulatory Affairs
Wm. Underwood Co.
One Red Devil Lane
Watertown, Massachusetts 02172

Dear Dr. Ebert:

Re: IBT No. 632-03040 - Mutagenic Study With Ac'cent Brand Monosodium-L-Glutamate in Albino Mice

We are submitting herewith our laboratory report dated May 9, 1973, prepared in connection with the above study.

Very truly yours,

J. C. Calandra
President

JCC: sjn

REPORT TO

WM. UNDERWOOD CO.

MUTAGENIC STUDY WITH AC'CENT BRAND MONOSODIUM-L-GLUTAMATE IN ALBINO MICE

MAY 9, 1973

IBT NO. 632-03040

I. Introduction

A mutation is a change in the character of a gene such that morphologic, physiologic, and/or biochemical alterations are produced. If this change in gene character occurs in the germinal cell, the alteration can be transmitted to succeeding generations. Changes of this nature can be artificially induced (irradiation, chemical exposure) or they may be spontaneous. A dominant lethal mutation, occurring in the male germinal cell, may lead to the inability of the affected cell to fertilize an egg or, once having fertilized, to the failure of development beyond the blastocyst stage (implantation). Male mice, treated with the test compound, are mated with untreated females. The numbers of pre-implantation losses and early resorptions in female mice, dissected at mid-gestation, are used to calculate the mutation rate based on the induction of dominant lethal mutations.

II. Summary

Male albino mice were treated with Ac'cent brand Monosodium-L-Glutamate (MSG, Batch No. 64047-1179). Treatment consisted of a single oral dose, via gavage, of MSG at levels of 2.7 (T-I) or 5.4 (T-II) g/kg of body weight. Effects on male germinal cells were monitored by mating treated animals with groups of 3 untreated females for each of 6 consecutive weeks. Females were sacrificed at mid-term of pregnancy. The uterus was exposed and carefully examined for signs of early embryonic death, which are observed to be deciduomata.

Animals in the T-I level had slightly lower mating indices than control animals. However, T-II mating indices were comparable to those of controls. The lowered T-I mating indices are not of biologic significance.

When sacrificed, females that had mated with treated males from either group had numbers of implantations, resorptions, and embryos similar to those of females that had mated with control males. Mutation rates, calculated from these values, for treated animals compared favorably with those for control animals.

Therefore, MSG administered as a single oral dose of 2.7 or 5.4 g/kg to male mice did not induce early embryonic death in utero in females that had mated with those males. Genetic damage, as manifested by dominant lethal mutations, did not occur.

Respectfully submitted,

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III. Procedure

A. Outline of Experiment

The test material was Ac'cent brand Monosodium-L-Glutamate (MSG, Batch No. 64047-1179). Charles River strain albino mice were received at this laboratory at 60 to 70 days of age for use in the study. The organization of groups is presented in Table I.

TABLE I

TEST MATERIAL: MSG

Mutagenic Study - Albino Mice

Organization of Groups

Group	Dose Level* (g/kg)	Number of Males Treated
C	-	12
T-I	2.7	12
T-II	5.4	12

* MSG was administered as a 27 percent solution in distilled water. Control males received the vehicle in amounts equivalent to those received by the T-II males.

B. Dosage Levels

The treatment levels were based on the acute oral LD_{50} value of 16.2 g/kg. The material was administered as a single oral dose (intubation) in solution with distilled water to male mice. Control animals received the vehicle in volumes equivalent to those given the high test group.

C. Mating Schedule

Each group consisted of 12 male mice, each of which was placed in a cage with 3 untreated virgin females immediately after dose administration. At the end of 1 week, the females were removed from the cage and replaced by another group of 3 females. This procedure continued for 6 consecutive weeks, a period of time required for maturation of the male mouse germ cells from the spermatocyte to the mature spermatozoon. Following the sixth week of mating, all males were sacrificed.

D. Female Sacrifice

The females were sacrificed approximately 1 week after removal from the breeding cage, at which time most females that had mated were at mid-pregnancy. All animals were sacrificed by carbon dioxide asphyxiation. The numbers of implantation sites, resorption sites, and embryos were recorded. Females were judged to be pregnant if corpora lutea were present in the ovaries.

Resorption sites were divided into 2 groups, early deaths (deciduomata) and late deaths. Late deaths refer to embryos which develop

to a relatively advanced stage prior to death, so that the placenta and fetal membranes or remnants thereof are visible. The frequency of late deaths is apparently unaffected by mutagens and, therefore, would appear to be non-genetic in this test system. Deciduomata occur at the sites of implanted blastocysts which fail to develop following implantation. The mutagenicity of the chemical can be measured by the proportions of all implantations which are deciduomata.

Mutagenicity can also be measured by comparing the mean number of viable embryos in the test group to the number obtained in the control group. Calculation of the mutation rate using this criterion assumes that pre-implantation losses among non-affected test animals will be essentially the same as losses observed among control animals.

IV. Results

A. Treatment Levels

The dose levels used in the study were based on the acute oral LD_{50} of 16.2 g/kg. T-II animals received 5.4 g/kg (0.333 x 16.2). T-I animals received 2.7 g/kg (0.5 x 5.4 or 0.167 x 16.2).

B. Mortality and Reactions

No deaths occurred and no unusual reactions were noted among treated or untreated animals.

C. Mating Performance

Mating indices, defined as the number of animals pregnant divided by the number of females mated times 100, and the number of surviving males for each post-treatment week are shown in Table II.

Mating indices were somewhat low for T-I animals at weeks 1, 2, and 3. However, they were not unusually low and since this was not observed among T-II animals, the significance of this finding is questionable. The mating indices for T-II animals compared favorably with those for control animals.

TABLE II

TEST MATERIAL: MSG

Mutagenic Study - Albino Mice

Mating Performance

Group	Test Week Number	Number of Surviving Males	Number of Animals Pregnant / Number of Females Mated	Mating Index (Percent)
C	1	12	24/36	66.7
	2	12	23/36	63.9
	3	12	26/36	72.2
	4	12	28/36	77.8
	5	12	27/36	75.0
	6	12	29/36	80.6
T-I	1	12	15/36	41.7
	2	12	16/36	44.4
	3	12	14/36	38.9
	4	12	19/36	52.8
	5	12	19/36	52.8
	6	12	21/36	58.3
T-II	1	12	21/36	58.3
	2	12	24/36	66.7
	3	12	24/36	66.7
	4	12	25/36	69.4
	5	12	25/36	69.4
	6	12	29/36	80.6

D. Sacrifice Data

Data pertaining to the numbers of corpora lutea, implantation sites, resorption sites (early and late differentiated), and embryos are presented in Table III. Laboratory data have shown that the untreated female mouse has a mean of 13.5 corpora lutea. Therefore, the number of pregnant animals is multiplied by 13.5 to obtain the calculated number of corpora lutea.

No differences were noted between data for test animals and data for control animals. The numbers of implantation sites, resorption sites, and embryos per female for test animals were essentially the same as those numbers for controls.

TABLE III

TEST MATERIAL: MSG

Mutagenic Study - Albino Mice

Summary of Sacrifice Data

Group	Test Week Number	Pregnant Animals Examined	Calculated Corpora Lutea	Implantation Sites	Resorption Sites Early	Resorption Sites Late	Embryos
C	1	24	324	275 (11.4)	12 (0.5)	2 (0.1)	261 (10.9)
	2	23	310	263 (11.4)	8 (0.3)	0 (0.0)	255 (11.1)
	3	26	351	294 (11.3)	13 (0.5)	3 (0.1)	278 (10.7)
	4	28	378	350 (12.5)	10 (0.4)	4 (0.1)	336 (12.0)
	5	27	364	320 (11.8)	18 (0.7)	5 (0.2)	297 (11.0)
	6	29	392	344 (11.9)	15 (0.5)	2 (0.1)	327 (11.3)
T-I	1	15	202	178 (11.9)	6 (0.4)	2 (0.1)	170 (11.3)
	2	16	216	165 (10.3)	7 (0.4)	1 (0.1)	157 (9.8)
	3	14	189	178 (12.7)	5 (0.4)	1 (0.1)	172 (12.3)
	4	19	256	240 (12.6)	10 (0.5)	1 (0.1)	229 (12.0)
	5	19	256	248 (13.0)	5 (0.3)	3 (0.2)	240 (12.6)
	6	21	284	266 (12.7)	18 (0.8)	1 (0.1)	247 (11.8)
T-II	1	21	284	287 (13.7)	13 (0.6)	0 (0.0)	274 (13.0)
	2	24	324	276 (11.5)	6 (0.2)	1 (0.1)	269 (11.2)
	3	24	324	323 (13.4)	11 (0.4)	2 (0.1)	310 (12.9)
	4	25	338	316 (12.6)	11 (0.4)	1 (0.1)	304 (12.2)
	5	25	338	314 (12.6)	10 (0.4)	2 (0.1)	302 (12.1)
	6	29	392	371 (12.8)	25 (0.9)	3 (0.1)	343 (11.8)

Note: Numbers in parentheses are mean values.

E. Mutagenic Data

A summary of results for the mutagenic study is presented in Table IV.

Pre-Implantation Loss is defined as:

$$\frac{\text{Number of Corpora Lutea - Number of Implantation Sites}}{\text{Number of Corpora Lutea}} \times 100$$

The mutation rate may be calculated by comparing the number of early resorptions (deciduomata) to the total number of implantations for each group.

$$\frac{\text{Number of Early Resorption Sites}}{\text{Number of Implantation Sites}} \times 100 \quad \text{(A in table)}$$

Another way of expressing the mutation rate, which takes into account pre-implantation losses, is by comparing the mean number of normal embryos in each test group to the mean number of embryos in the control group.

$$100 - \left(\frac{\text{Embryos Test Group/Female}}{\text{Embryos Control Group/Female}} \times 100\right) \quad \text{(B in table)}$$

The values presented for each group were obtained by comparing the mean values of that group to the mean values for the contemporary control group (a in table) and to cumulative control data (b in table). Values with a minus (-) sign indicate that the mean number of embryos for that group and week was greater than that of the comparative control.

Mutation rates and the number of pre-implantation losses among MSG treated animals are not unusual and give no indication of a mutagenic response.

TABLE IV

TEST MATERIAL: MSG

Mutagenic Study - Albino Mice

Summary of Mutagenic Data

Group	Test Week Number	Pre-Implantation Loss (Percent)	Mutation Rates A	B a	B b
C	1	15.1	4.4	-	5.2
	2	15.2	3.0	-	2.6
	3	16.2	4.4	-	7.0
	4	7.4	2.8	-	-4.3
	5	12.1	5.6	-	5.2
	6	12.2	4.4	-	2.6
T-I	1	11.9	3.4	-3.7	1.7
	2	23.6	4.2	11.7	14.0
	3	5.8	2.8	-15.0	-7.0
	4	6.2	4.2	0.0	-4.3
	5	3.1	2.0	-14.5	-8.6
	6	6.3	6.8	-4.4	-1.7
T-II	1	0.0	4.5	-19.3	-13.0
	2	14.8	2.2	-0.9	2.6
	3	0.3	3.4	-20.6	-12.2
	4	6.5	3.5	-1.7	-6.1
	5	7.1	3.2	-10.0	-4.3
	6	5.4	6.7	-4.4	-1.7

31

ansplacental Ratios of Serum Free Amino cids During Pregnancy in the Rhesus Monkey

orge R. Kerr, M.D.* and Harry A. Waisman, M.D.*

The presence and sequelae of a disturbance in amino acid metabolism which occurs during postnatal life may be defined by appropriate physical, chemical and psychological measurements. There are no criteria, however, for defining the presence and sequelae of the same disturbance occurring during prenatal life. This lack of knowledge is a major impediment to our understanding of amino acid metabolism because at no other time in life does the rate of protein synthesis reach the peak that is evident during fetal development [1, 2].

Until recently there was no simple way of obtaining information on human fetal development. The diversities of nature become more obvious as one steps outside the biologic boundaries surrounding each species and order, and lower species differ enough from the human in terms of placental physiology and rates of fetal growth to seriously reduce their value for defining mechanisms of amino acid metabolism which would be meaningful in terms of human fetal biology. Fortunately, the converse is also true and data derived from one species are often comparable to those observed in another species of the same order. This provides us with a means for avoiding the moral and procedural objections which render the human an unlikely source of the basic information needed in the area of fetal biology, and for substituting, in his place, a subhuman primate. We have concentrated our investigations into the definition of normal growth and development in the rhesus monkey (*m. mulatta*) and this report presents the transplacental ratios for serum amino acids during pregnancy in this species.

METHODS

Female rhesus monkeys menstruate approximately every 28 days. Ovulation usually occurs on the eleventh day after the onset of the cycle, and females in our colony are bred only on this day so that the gestational age may be accurately

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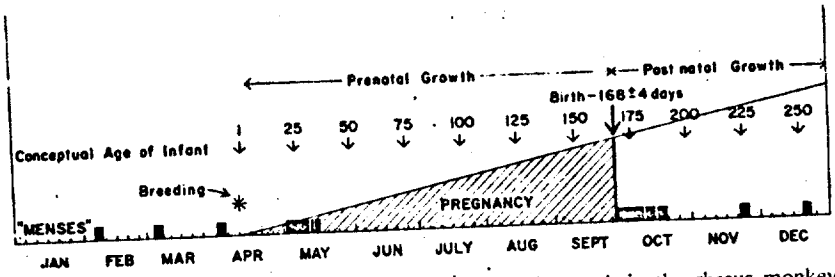

FIG. 31-1. Schematic diagram of prenatal and postnatal growth in the rhesus monkey. *Arrows* indicate the conceptual ages chosen for study. Vaginal bleeding is usually noted about five weeks after conception as a prolonged menses.

known. Products of conception are delivered by cesarean section at exactly 50, 75, 100, 125 and 150 days of age or are sacrificed at specific ages in postnatal life. A summary of this experimental approach is indicated in Figure 31-1.

Fetal and maternal levels of all serum free amino acids were studied during pregnancy at the ages indicated in Table 31-1. At cesarean section delivery, simultaneous

Table 31-1

Number of Studies at Each Gestational Age

Gestational Age	Number of Pregnancies
50	3
75	5
100	4
125	3
150	3
168 ± 4	8*

*Full-term vaginal delivery

blood samples were drawn from the umbilical vein of the infant and the maternal inferior vena cava. These were allowed to stand at 5°C for four hours, centrifuged, and the serum separated. All samples are analyzed for amino acids within five days by the Gerritsen et al. [3] modification of the method of Spackman, Stein and Moore [4].

RESULTS

The cord:maternal ratios (C:M) for all amino acids at full-term pregnancy in the rhesus monkey are indicated in Figure 31-2. It is apparent that the fetal level exceeded the maternal level in all cases. In attempting to define the mechanisms responsible for the elevated C:M at full-term pregnancy, the serum levels of free amino acids were first compared between the umbilical cord samples and those from a group of ten one-year-old macaques (Table 31-2). Except in the cases of taurine, alanine, lysine and 3-methyl-histidine, the fetal levels were not significantly elevated

Table 31-2

Serum Amino Acid Changes* in Eight Full-Term Fetuses Compared to the Levels in Ten One-Year-Old Monkeys

Elevated	*Unchanged*	*Lowered*
Taurine*	Glutamine plus glutamic acid	Tyrosine*
Alanine*	Glycine	Serine*
Lysine*	Aspartic acid plus threonine	Arginine
3-Methyl-histidine*	Proline	
	Valine	
	Leucine	
	Phenylalanine	
	Isoleucine	
	Histidine	

* Significant at $p = < .01$

The free amino acids in venous blood of eight adult non-pregnant female monkeys and eight females at full-term delivery were next compared (Table 31-3). 3-Methyl-histidine was the only amino acid significantly elevated at full-term pregnancy although taurine and lysine were also higher than in the non-pregnant controls.

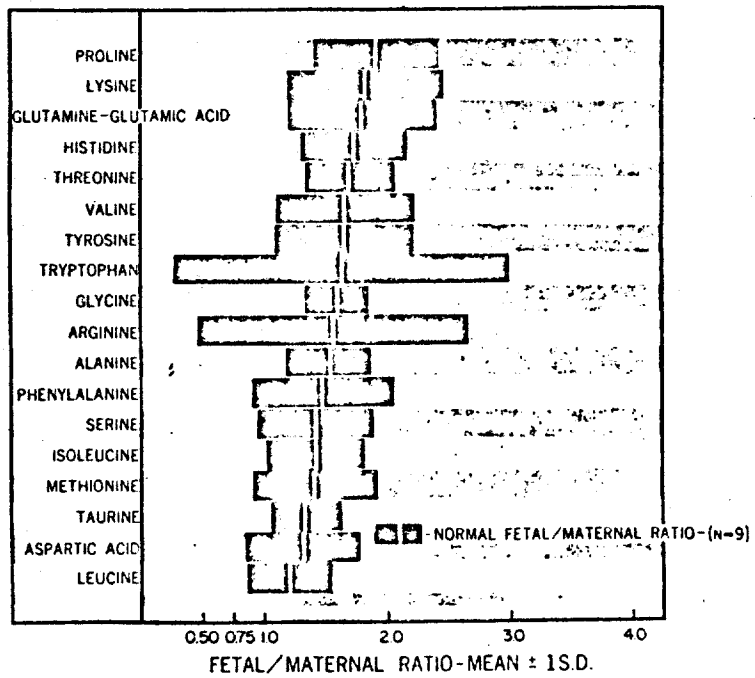

FIG. 31-2. Transplacental ratios (C:M) for amino acids at full term pregnancy in the rhesus monkey. The mean values (± 1 S. D.) from nine pregnancies are shown.

Table 31-3

Serum Amino Acid Changes* in Eight Full-Term Pregnant Females Compared to Eight Non-Pregnant Female Monkeys

Elevated	*Unchanged*	*Lowered*
3-Methyl-histidine*	Valine	Glutamine plus glutamic acid*
Taurine	Arginine	Proline*
Lysine		Glycine*
		Serine*
		Aspartic acid plus threonine*
		Leucine*
		Tyrosine*
		Histidine*
		Alanine
		Methionine
		Phenylalanine
		Isoleucine

* Significant at $p = <.01$

With the exception of valine and arginine, all other amino acids were lower than in controls. It appears that in this species, the elevated C:M for amino acids was caused, in all but four instances, by low maternal values. The C:M for taurine, lysine and 3-methyl-histidine were the result of elevated fetal levels. The C:M for alanine was produced by both a reduction in maternal and an increase in fetal values.

Data from human pregnancies indicate that the transplacental ratios for amino acids are highest in the most immature fetus [5]. In these studies, by 75 days of

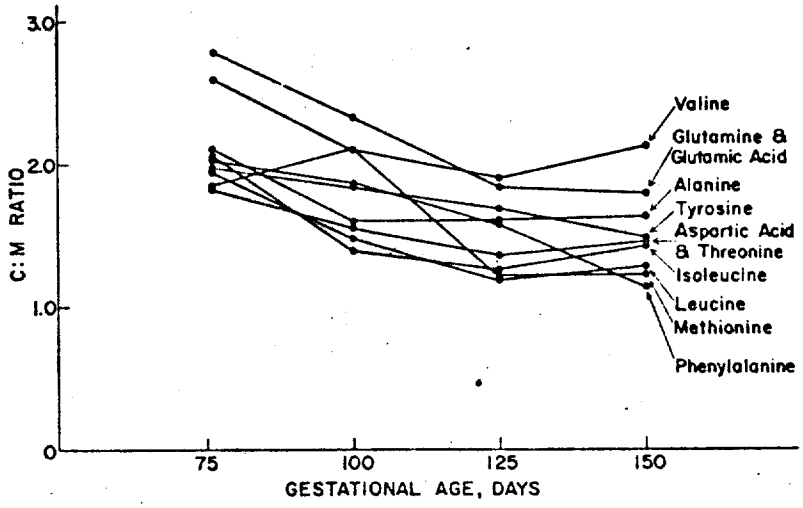

FIG. 31-3. Transplacental ratios (C:M) for amino acids during pregnancy in the rhesus monkey.

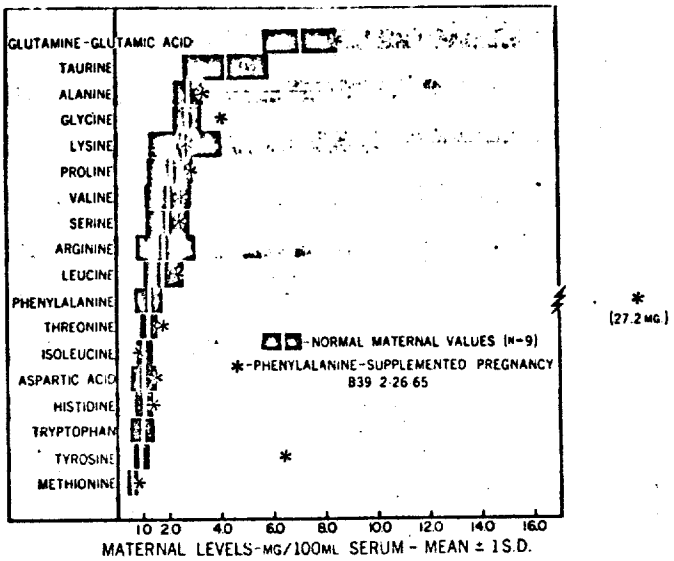

FIG. 31-4. Maternal serum free amino acids at full-term pregnancy in the rhesus monkey. *Solid blocks* indicate the mean values (± 1 S. D.) from control pregnancies. *Asterisks* indicate values from one pregnant female fed a diet supplemented with excess L-phenylalanine.

pregnancy, the maternal levels of amino acids were as low as those observed at full term. Samples collected at 100, 125 and 150 days of gestation did not indicate a further change in the maternal levels. On the other hand, fetal levels were often higher in early pregnancy and accounted for a trend to higher C:M at those ages. This was evident for those amino acids indicated in Figure 31-3. The wide range of values at each age obviated further consideration of the significance of these changes.

For the past few years cases have been reported in which women with phenylketonuria have given birth to mentally retarded, but non-phenylketonuric infants [6]. This finding implied that the high maternal level of phenylalanine (or its metabolites) was able to cross the placenta and in some way interfere with the normal growth of the fetal brain. The sequelae of maternal hyperphenylalaninemia during pregnancy could be investigated in the rhesus monkey model described above with some assurance that the data would be pertinent to pregnancy in the human. A group of pregnant female monkeys was therefore fed a milk diet supplemented with high levels of phenylalanine (1-2 g kg day), and elevated serum phenylalanine levels were consistently observed [7]. A typical maternal serum amino acid pattern obtained at the time of full-term vaginal delivery is shown in Figure 31-4. Except in the case of tyrosine, the elevated maternal phenylalanine level was obtained without interfering with the serum levels of any other amino acid.

The C:M for phenylalanine at full-term pregnancy in this species is approximately 1.5; the ratio for tyrosine approximately 1.8. The level of amino acids found in the umbilical cord serum of the fetus born to this monkey mother is indicated in Figure 31-5. The mother's phenylalanine level was 27.2 mg 100 ml; the cord level was 43.7 mg/100 ml. The mother's tyrosine level was 6.2 mg 100 ml; the cord tyrosine level

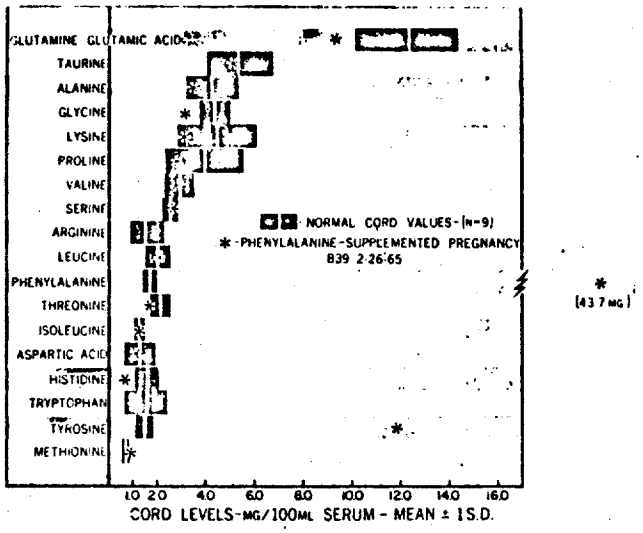

FIG. 31-5. Umbilical cord serum free amino acids at full-term pregnancy in the rhesus monkey. *Solid blocks* indicate the mean values (± 1 S. D.) from control cord sera. *Asterisks* indicate values from the cord serum of the infant born to the mother described in Figure 31-4.

was 11.9 mg/100 ml. All other amino acids of cord serum were within the normal range, and the C:M for all amino acids, including phenylalanine and tyrosine, were also within the normal range.

DISCUSSION

Studies using pregnant rhesus monkeys have shown that placental structure and physiology are comparable to those observed in the human. Growth of the fetus is defined in terms of weight in Figure 31-6, and shows the type of data which are obtained from these studies. This is the simplest expression of growth; the same type of graph can be constructed for linear growth, cell diameters, tissue composition and other parameters of both structural or functional development. While this curve suggests that the maximum rate of fetal growth occurs during the middle third of pregnancy, in actuality it occurs during the first few weeks of fetal life [8]. Figure 31-7 indicates that the 50-day-old fetus consists of an estimated one-half million cells and the 150-day-old fetus consists of about one-half billion cells. Beginning from one fertilized ovum, total fetal growth can be accounted for by exactly 30 generations of binary cell divisions. The intriguing fact is that by 50 days of fetal age, when only 1 per cent of the total fetal weight has been accumulated, over 60 per cent of the cell divisions have already occurred. It is hardly surprising that a teratogenic drug or virus may cause havoc by interfering with this almost malignant rate of growth.

It has been known for some time that the mammalian placenta delivers amino acids to the fetus by means of an active transport process, specific for the L-amino

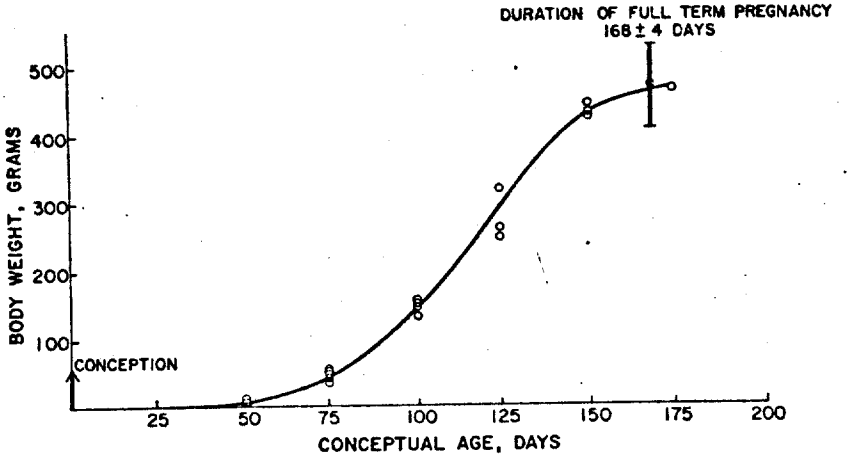

FIG. 31-6. Change in body weight during fetal life of the rhesus monkey. The *solid bar* indicates the birthweight (mean ± 1 S. D.) at spontaneous vaginal delivery.

acids [9]. The fact that amino acids in fetal blood were higher than those in the maternal organism was first noted in 1917 by Morse et al. [10], and Lichtenstein [11] later reported that the highest levels were observed in the most immature fetus. Christensen et al. [12] reported in 1957 that the reduced maternal levels of plasma amino acids which were found during human pregnancy were correlated with an increased urinary excretion of amino acids. Ghadimi and Pecora [5] noted that the ratios of all free amino acids in umbilical cord plasma to those in maternal plasma were greater than 1. They concluded that this was brought about concurrently by

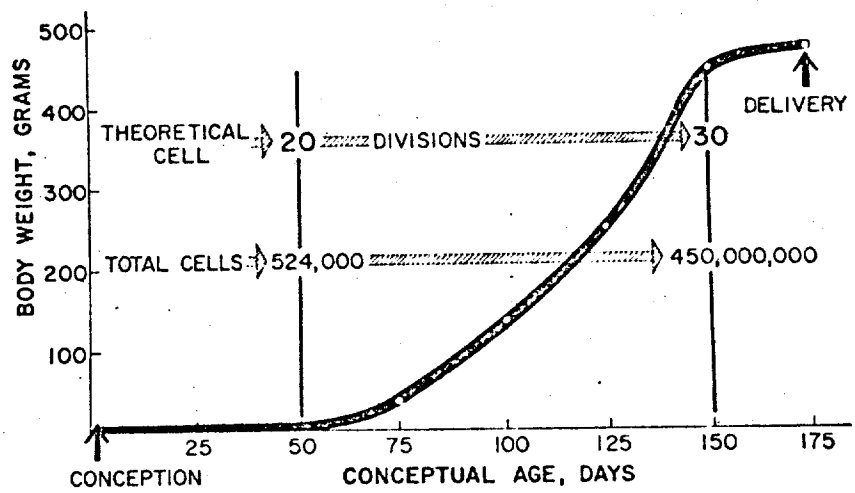

FIG. 31-7. Theoretical analysis of fetal growth in the rhesus monkey.

reduced maternal, and increased fetal amino acid levels. The highest fetal:maternal ratios were observed in association with prematurity.

Our data indicate that only for lysine, alanine, taurine and 3-methyl-histidine is there an elevation in the free amino acids of umbilical cord serum. For all other amino acids, the elevated C:M is caused by reduced maternal values. While maternal levels did not appear to change throughout pregnancy, fetal levels in early gestation were often higher than those in older fetuses.

The experiment with monkeys fed phenylalanine-supplemented diets during pregnancy indicated that the placental mechanism continued to produce higher fetal phenylalanine levels than were present in the mother. If phenylalanine per se (or its metabolites) is responsible for "brain damage" in the phenylketonuric human, it is probable that children born to mothers with this disorder would have been exposed to even higher phenylalanine levels. The intriguing fact is that this fetal "damage" would be produced by a normal physiologic placental mechanism.

In the past we have attributed to the placenta the role of "protecting" the fetus from adverse biochemical insults. It now appears that we can do so no longer. The placenta is a superb organ, but can only perform a given function when confronted with a compound of given physicochemical configuration. In the case of amino acids, the placenta functions to maintain a higher blood level in the fetus than in the mother; it will do this regardless of the maternal level. This mechanism is ideally suited to periods of maternal deprivation, but if a pregnant primate female has an elevated serum level of free amino acids the placenta will efficiently magnify this abnormality in the fetal circulation. Infants born to these mothers were normal in terms of size, weight and neurologic examination at birth, and were subsequently raised to the best of our ability on a control diet, in an attempt to define whether intellectual or social sequelae would have resulted from this experiment. Although the psychological tests have not been completed, preliminary results make it apparent that a statistically significant defect in the ability to learn is present in all infants from hyperphenylalaninemic mothers. Of perhaps greater significance is the observation that while these infants eventually learn the simple tasks involved in the intelligence tests, they have not learned how to properly socialize with other infant monkeys.

The implications of these results merit further attention because many mild defects in amino acid metabolism may be present in seemingly healthy pregnant women. Whether any of these mild abnormalities could do any "harm" by producing even greater abnormalities in the fetus is a matter for speculation. At least 3 per cent of all human deliveries show some degree of mental retardation and in the vast majority of cases we do not know how brain damage is initiated except that it probably occurs during fetal or perinatal life. It is entirely possible that some types of retardation are due to the normal but potentially dangerous placental mechanism just demonstrated.

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SHORT COMMUNICATION

Lack of an effect of dietary monosodium-L-glutamate on some glutamate-metabolizing enzymes in developing rat brain

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A NUMBER of reports have appeared which relate the parenteral administration of monosodium-L-glutamate (MSG) to infant animals of several species with acute necrosis of neuronal populations in the retina (LUCAS and NEWHOUSE, 1957; POTTS, MODRELL and KINGSBURY, 1960; COHEN, 1967; OLNEY, 1969*a*) and hypothalamus (OLNEY, 1969*b*; OLNEY and SHARPE, 1969; AREES and MAYER, 1970; OLNEY, 1971). Only one report has appeared indicating that such lesions are in evidence following oral intake of MSG by mice (OLNEY and HO, 1970). A conflicting paper recently reported the absence of morphological changes in the hypothalamic regions of infant monkey brains when these animals were fed MSG by stomach tube (REYNOLDS, LEMKEY-JOHNSON, FILER and PITKIN, 1971).

Studies have been conducted in our laboratory to establish effects of dietary MSG on certain constituents of brain and liver. Weanling rats were fed diets containing as much as 20% (w/w) MSG for 16 weeks, and brains were then analyzed for a number of glutamate-related metabolites; the only significant change was a decrease in GABA (PROSKY and O'DELL, 1971). This type of analytical approach was followed for four generations with control rats fed Purina chow and with treated rats fed the chow supplemented with 10% (w/w) MSG. The results of analyses of brains of second-generation neonatal rats showed that oral intake of MSG had little effect on the substrates measured and no effect on the activity of glutamic decarboxylase (GAD; L-glutamate 1-carboxylase, EC 4.1.1.15), the enzyme which catalyses the decarboxylation of L-glutamic acid to GABA (PROSKY and O'DELL, 1971).

We report here the effects of dietary MSG on the activity of three enzymes in the brains of our fourth-generation rats. The enzymes studied were glutamic-pyruvic transaminase (GPT; L-alanine : 2-oxoglutarate aminotransferase, EC 2.6.1.2), glutamic-oxaloacetic transaminase (GOT; L-aspartate : 2-oxoglutarate aminotransferase, EC 2.6.1.1) and glutamic dehydrogenase (GDH; L-glutamate NAD oxidoreductase, EC 1.4.1.2). Brain weights and total protein were also determined.

MATERIALS AND METHODS

Holtzman albino weanling rats were fed a Purina laboratory chow diet (controls) or this diet supplemented with 10% (w/w) MSG for 100 days (treated). Twenty male and 20 female rats in each of the two groups were mated in separate cages, on a one to one basis, for a 7-day period and the resultant offspring (first generation, F_1) were continued on the same diet as their parents for 100 days. This same procedure was continued until the birth of the F_4 generation. At this time six to eight neonatal pups from each group were sacrificed on days 1, 3, 5, 10 and 21. After the rats were decapitated, the brains were rapidly removed, weighed and homogenized in 10 vol. of ice-cold glass-distilled water in a Teflon–glass homogenizer for 2 min. Methods used to determine enzymatic activities were based on the oxidation of NADH; the methods of BERGMEYER and BERNT (1965a; 1965b) were used for GOT and GPT, and the method of SCHMIDT (1965) was used for GDH. All enzyme assays were carried out on the day of sacrifice. Protein was determined by the colorimetric method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951).

Enzyme activities are expressed in units; 1 unit decreased the optical density of NADH at 366 nm by 0·001 in 1 min at 25°C.

Abbreviations used: MSG, monosodium-L-glutamate; GAD, glutamic decarboxylase; GPT, glutamic-pyruvic transaminase; GOT, glutamic-oxaloacetic transaminase; GDH, glutamic dehydrogenase.

TABLE 1.—EFFECT OF DIETARY MSG ON BODY WEIGHTS, BRAIN WEIGHTS AND PROTEIN CONTENT, AND ACTIVITIES OF GOT, GPT AND GDH IN BRAINS OF FOURTH-GENERATION RATS

Group	Body weight (g)	Brain weight (g)	Protein/brain (mg/g)	GOT activity (units)/protein mg	GPT activity (units)/mg protein	GDH activity (units)/mg protein
			1-day-old			
Control	6·48 ± 0·16	0·2631 ± 0·0038	78·7 ± 1·2	141 ± 6	6·2 ± 0·4	2·16 ± 0·35
Treated	5·98 ± 0·20	0·2712 ± 0·0048	75·8 ± 1·1	147 ± 4	6·6 ± 0·8	3·28 ± 0·77
			3-days-old			
Control	7·61 ± 0·16	0·3549 ± 0·0039	75·2 ± 0·8	153 ± 7	8·1 ± 1·7	1·62 ± 0·38
Treated	9·41 ± 0·20*	0·3992 ± 0·0101*	73·0 ± 0·6	183 ± 4*	7·4 ± 0·4	1·64 ± 0·38
			5-days-old			
Control	11·47 ± 0·28	0·5129 ± 0·0073	74·8 ± 1·4	187 ± 8	8·2 ± 0·6	3·40 ± 0·36
Treated	12·54 ± 0·40*	0·5681 ± 0·0148*	74·6 ± 1·0	178 ± 7	8·3 ± 0·6	3·27 ± 0·49
			10-days-old			
Control	20·87 ± 0·54	0·9191 ± 0·0116	83·9 ± 1·6	248 ± 9	10·6 ± 0·8	3·15 ± 0·40
Treated	22·25 ± 1·38	0·9539 ± 0·0289	82·3 ± 0·8	252 ± 8	10·3 ± 0·4	2·40 ± 0·26
			21-days-old			
Control	49·20 ± 1·71	1·4027 ± 0·0270	107 ± 2	458 ± 14	14·1 ± 0·8	4·97 ± 0·54
Treated	55·09 ± 2·64	1·4980 ± 0·0278*	110 ± 1	360 ± 16*	15·2 ± 0·6	4·64 ± 0·56

Each value represents the mean ±S.E.M. of analyses on 6–8 brains.
Enzyme activity is expressed in units, as defined in text section on methods.
* Mean differs significantly from that of control group, $P < 0·01$.

RESULTS AND DISCUSSION

Effects of dietary MSG on brain levels of GOT, GDH and GPT are summarized in Fig. 1. The total activity of each of these glutamate-metabolizing enzymes rose sharply during the first 21 days of postnatal development. A 20-fold increase was observed for GOT and GPT activity while GDH activity increased by approximately 15-fold during the neonatal period. The magnitude of the increases resembles that previously reported for GAD (PROSKY, O'DELL and JOHNSON, 1971), the fourth major enzyme system involved in glutamate metabolism; however, no significant differences were noted between control and treated groups.

Body and brain weights of treated rats were significantly higher than those of controls ($P < 0.01$; Student t-test) at various times during the first 21 days of neonatal development (Table 1). The tendency of pups of parents fed MSG to increase brain and body weights more rapidly than controls during the neonatal period had been noted earlier and was ascertained to be a transient effect which disappeared as the animal matured (PROSKY *et al.*, 1971).

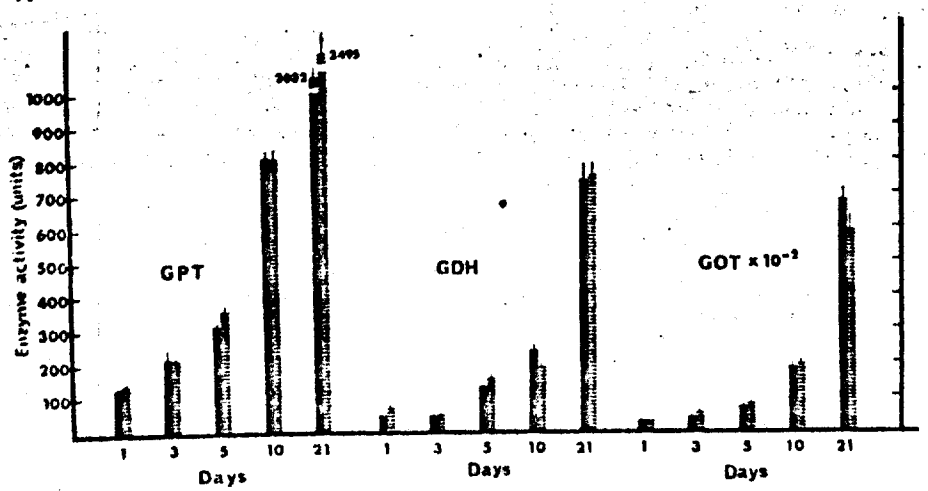

FIG. 1.—Activities of GOT, GPT and GDH in whole brain homogenate of control (solid bars) and treated (lined bars) rats. These values were determined from initial reaction rates over a 10-min period when the change in optical density, measured at 5-s intervals with a Gilford 2000 multiple sample spectrophotometer, is linear. Vertical lines = S.E.M.

From days 1 through 21 the protein content of the brain reflected the growth of the organ, both increasing by approximately 6-fold. Although no significant differences in brain protein content were noted between treated and control rats, it is important to note that during this same period the enzymes responsible for the metabolism of glutamate were developing at 2–3 times the rate of total protein synthesis in the brain (Table 1). A possible explanation for the pathogenic action of excess MSG may derive from its ability to increase ionic permeability and increase intracellular water accumulation (KUHAR, 1971). The rapid development of high activities of transaminase, oxidase and decarboxylase enzyme systems in the brain which metabolize glutamate would probably preclude accumulation of glutamate in this organ. The present results agree with our earlier findings in which rats fed levels as high as 20% MSG maintained brain levels of glutamate comparable to those of controls at 9 μmol/g of brain (PROSKY and O'DELL, 1971).

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BRAIN DAMAGING POTENTIAL OF PROTEIN HYDROLYSATES

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ABSTRACT

Casein and fibrin hydrolysates commercially available for use in human parenteral alimentation therapy were administered subcutaneously to 10 day old mice at 5 doses (20, 40, 60, 80 and 100 microliters/g body wt). Casein hydrolysate at each dose tested and fibrin hydrolysate at 80 and 100 microliters/g induced acute degeneration of neurons in the developing hypothalamus. Hypothalamic lesions were identical to those which occur in various animal species following oral or parenteral administration of acidic amino acids such as glutamic, aspartic and cysteic acids. A control amino acid mixture excluding glutamic, aspartic and cysteic acids induced no brain damage in these experiments. It is suggested that a relatively low combined concentration of acidic amino acids is one of the criteria a parenteral alimentation preparation should meet in order to be maximally safe as well as nutritionally effective.

Total parenteral nutrition for patients who cannot be nourished through the gastrointestinal tract is being employed in hospitals with increasing frequency, particularly for surgical and pediatric patients,[1-3] including the premature.[3,4] Protein digests, prepared either by enzymatic hydrolysis of casein or acid hydrolysis of fibrin, provide a relatively inexpensive source of amino acids for this form of therapy. There are striking differences, however, in the free amino acid composition of hydrolysate preparations in current use. For example, Stegink and Baker reported[5] that Amigen^R, a casein preparation contains over 5 times as much glutamic acid (1960 micromoles/100 ml) as the fibrin hydrolysate, Aminosol^R (358 micromoles/100 ml). On the other hand, Aminosol^R contains 112 micromoles/100 ml of another acidic amino acid, cysteic acid while Amigen^R contains none. The content of a third acidic amino acid, aspartic acid is more nearly equal in the two preparations (Aminosol^R 660 micromoles/100 ml; Amigen^R 500 micromoles/100 ml). For purposes of the present study it is noteworthy that the combined concentration of acidic amino acids in Amigen^R (2460 micromoles/100 ml) is more than double that in Aminosol^R (1130 micromoles/100 ml).

The present study focuses specifically on the acidic amino acids contained in protein hydrolysates because of recent findings that certain acidic amino acids, including glutamic, aspartic and cysteic acids have unique brain damaging properties.[6-9] The most extensively studied compound in this group, glutamic acid - usually given as the sodium salt, monosodium glutamate (MSG) - has recently been shown by numerous investigators to induce hypothalamic damage when given to immature animals.[6-22]

Susceptibility to MSG-induced hypothalamic damage has been demonstrated in several species, including rhesus monkeys,[13,18] following either oral[7,10-14] or subcutaneous[6,10,11,15,16] doses in the range of 0.5 to 1 mg/g of body weight. Rodents treated with MSG in the first 10 days of life develop obesity,[6,17,23,24] neuroendocrine disturbances[6,23] and possible learning deficits[25] in later life. It has also been shown that subcutaneously administered glutamate gains entry to and is selectively accumulated by the arcuate nucleus of infant mouse hypothalamus, a process which parallels in time course the destruction of neurons in that nucleus.[20] The molecular specificity of MSG-induced brain damage is such that it is reproduced by subcutaneous administration of a select group of other acidic amino acids (aspartic, cysteic, cysteine sulfinic, homocysteic acids and certain of their substituted synthetic analogues) which share with glutamate an ability to excite neuronal firing when introduced by microelectrophoresis into the mammalian brain.[7-9,16] An additive neurotoxic effect is produced by subcutaneous administration of any of the "neuroexcitatory" amino acids in combination with one another to immature mice.[7,9]

Since protein hydrolysates currently in use contain substantial concentrations of free acidic amino acids we postulated that the parenteral administration of these preparations to infants might entail risk of brain damage. Further, if the total acidic amino acid content of casein hydrolysates substantially exceeds that of fibrin preparations, the former might produce more severe brain damage than the latter at any given exposure level. The present study was undertaken to test these hypotheses. Protein hydrolysates currently being used for parenteral alimentation of human

infants were the preparations tested, but for obvious reasons, experimental animals rather than human infants were chosen as test subjects.

METHODS AND MATERIALS

A total of 120 Webster Swiss albino mice (National Animal Laboratories, St. Louis, Mo.), 10 days old were studied. Ninety experimental animals were given single subcutaneous injections of one of three protein hydrolysate preparations: (1) Travamin^R, casein enzymatic digest, Travenol Laboratories, Inc. (previously called Amigen^R). (2) CPH^R casein enzymatic digest, Cutter Laboratories, Inc. (3) Aminosol^R, fibrin hydrolysed by acid, Abbott Laboratories. Each solution was given undiluted as commercially prepared (5% hydrolysate/5% dextrose) to 6 animals at one of 5 doses (20, 40, 60, 80, or 100 microliters/g of body weight); 30 animals in all being treated with each solution.

Thirty additional mice, 6 being treated at each of the above doses, were given a control solution constituted as follows: (mg/100 ml) L-lysine 800, L-tryptophane 100; L-phenylalanine 200, L-methionine 200, L-threonine 400, L-leucine 1200, L-isoleucine 400, L-valine 320, L-arginine 580, L-histidine 200, glycine 400, L-tyrosine 200, potassium metabisulfite 60, potassium chloride 51, dextrose-5000; the rationale for the control being that it excludes the acidic and sulphur amino acids which have been shown to have brain-damaging properties while otherwise simulating the makeup of experimental solutions. Although the peptide content of the experimental solutions (protein hydrolysates are nearly 50% peptides) could not readily be duplicated, we attempted to compensate for this by increasing the concentrations of amino acids used in the control solution. Instead of being

5% hydrolysate (amino acids plus peptides), the control solution was 5% amino acids. The control solution was adjusted to pH 7.0 with HCl to bring it into the pH range of experimental solutions. All solutions were administered by injection under the skin of the back (Hamilton microliter syringe and 30 gauge needle) and the mice were housed in individual open containers under a lamp providing a constant ambient temperature of 31°C during the interval between treatment and sacrifice.

Five hours following injection both control and experimental mice were anesthetized with chloral hydrate and sacrificed by perfusion fixation as described elsewhere.[7,14] Processing of brain tissue for alternative examination by either light or electron microscopy was performed by methods previously described.[13,14] For purposes of evaluating lesion severity, the portion of the hypothalamus containing arcuate nucleus (nucleus periventricularis arcuatus hypothalami)[26] was serially sectioned and necrotic neurons were counted in a representative section (1 μm thick) cutting across the nucleus at its level of maximal damage. As illustrated elsewhere[7,8,13,14,18] the process of MSG-induced neuronal necrosis is characterized by coarse clumping and condensation of nuclear chromatin accompanied by massive perikaryal swelling. Neurons thus affected are readily distinguished by light microscopy from healthy neurons, provided brains are prepared by appropriate histological techniques and examined in the acute post-treatment interval, 3-6 hours after treatment being optimal.

RESULTS

The number of mice affected and the severity of hypothalamic damage at each dose of the administered solutions is given in Table 1. None of

the pups injected with the control solution at any dose sustained hypothalamic damage. None of those injected with the fibrin hydrolysate, Aminosol^R at 20, 40, or 60 microliters/g sustained lesions. Of those treated with Aminosol^R at 80 and 100 microliters/g, however, 1/3 and 1/2 respectively did have lesions which although small, were clearly of the type characteristically seen in MSG-treated animals. The casein hydrolysates CPH^R and Travamin^R were essentially equal in neutotoxic potency with both being substantially more potent than the fibrin preparation. At the lowest dose tested (20 microliters/g) only one animal was affected by the casein hydrolysates but 2/3 of those treated by either casein preparation were affected by the next dose (40 microliters/g) and all of the animals treated by either preparation at 60, 80 or 100 microliters/g were affected. A linear dose-response relationship was observed for each hydrolysate with lesion severity increasing steadily with each dose increment. Fig. 1 illustrates the normal appearance of the arcuate region of the hypothalamus in an unaffected control brain for comparison with a small lesion in the arcuate region of an animal given a high dose of fibrin hydrolysate (Fig. 2) and a large lesion in the arcuate region of one given a high dose of casein hydrolysate (Fig. 3).

Neither the animals receiving hydrolysate solutions nor those given the control preparation manifested acute symptoms of distress other than periodic restlessness and helpless maneuvering about as is characteristic of rodent pups isolated from the maternal nest. Experimental animals did not differ from controls in these non-specific behaviors.

DISCUSSION

Stegink and Baker[5] recently measured amino acid blood levels in 6 human infants receiving parenteral alimentaion therapy and reported no significant elevation of blood glutamate or aspartate levels while infusions of either fibrin or casein hydrolysates were being given. The authors concluded "It is difficult to imagine glutamate-induced neuronal damage without substantial elevation of plasma glutamate levels . . ." It may be questioned, however, whether the amino acid load being delivered to the brain of an infant on parenteral therapy would be reflected in measurements performed on peripheral venous blood. A venous blood sample would be expected to reflect the amino acid makeup of that portion of the circulating blood which had passed through the body tissues and was returning to the infusion site (superior vena cava). Thus, it could provide a baseline for glutamate levels just prior to the introduction of infusate into the system but does not reveal to what extent the introduction of infusate altered glutamate concentrations in that portion of the blood leaving the heart to recirculate through the tissues. The arcuate nucleus of the hypothalamus lies at the base of the brain where it is surrounded by the circle of Willis. Its rich arterial supply, not far removed in the circulatory system from the infusion site, might very well provide it with higher concentrations of acidic amino acids than venous blood levels would lead one to suspect. The striking tendency of arcuate nucleus to accumulate glutamic[20] and possibly other acidic amino acids, even against a steep tissue to blood concentration gradient, might result in these compounds being siphoned off and accumulated

in that hypothalamic region if even moderately elevated levels are being continuously circulated through it.

Absence of overt signs of acute toxicity in infant humans receiving protein hydrolysate infusions has been advanced as evidence that human infants are invulnerable to glutamate-induced brain damage.[27] However, evidence that immature monkeys[13],[18] as well as rodents undergo brain damage from doses of glutamate well below those required to produce acute symptoms and the present demonstration that mice pups sustain hydrolysate-induced brain damage without manifesting overt signs of an acute CNS disturbance tend to invalidate this argument.

Vomiting, a well recognized side effect of protein hydrolysate infusions, is generally attributed to the glutamic (and aspartic) acid content of these preparations.[28-30] If the relationship between glutamate-induced vomiting and brain damage were such that the former always preceded the latter, the occurrence of vomiting would be a useful warning signal. Unfortunately experiments with rhesus monkeys[13] indicate that rhesus infants, particularly premature infants, have a higher threshold for glutamate-induced vomiting than adults while the converse is true for glutamate-induced brain damage. Thus vomiting occurs in infant monkeys only at doses of glutamate which exceed those required to produce brain damage. If humans parallel monkeys in this respect, vomiting by a human infant from an overload of glutamate would not be a warning signal that brain damage is about to occur; it would be a reminder that silent damage to the infant hypothalamus may have already occurred. For purposes of evaluating risk, the subcutaneous dose of casein hydrolysate required to destroy nerve cells in the immature mouse hypothalamus

(20-40 ml/kg) and the rate at which a human infant might receive such a preparation intravenously (120 ml/kg/day)[5] cannot be directly compared since different routes of administration are involved. The general conclusion, however, that parenteral alimentation, particularly with casein hydrolysates, affords the human infant less than a wide margin of safety against hypothalamic damage seems warranted.

Although total parenteral therapy for infants with serious and sometimes life-threatening disorders is of unquestionable value, it is important to give continuing attention to possible means of improving the composition of the parenteral solutions used in this form of therapy. The amino acids which destroy hypothalamic neurons are not essential amino acids. In the absence of any nutritional requirement that they be introduced in high concentrations into the blood stream, limiting their concentrations in parenteral alimentation preparations may be advisable. A step in this direction would be achieved by simply electing to use fibrin instead of casein hydrolysates. Selectively removing the acidic amino acids from hydrolysates by absorbing them on ion exchange resins as suggested by Elwyn and Greenstein[31] is worthy of consideration. Amino acid solutions constituted from crystalline amino acids are currently being developed and may, in the future, be the products of choice since the concentration of one amino acid relative to others can readily be manipulated in such synthetic preparations. According to Heird et al.[32] either glutamic and aspartic acids or other anions which consume hydrogen ion such as acetate or lactate must be included in parenteral alimentation formulas for human infants to prevent metabolic acidosis from developing.

Our findings suggest that the combined total concentration of acidic amino acids should be kept at a relatively low level to provide an adequate margin of safety against their brain damaging potential. Maintaining the concentration of glutamic and aspartic acids at relatively low levels while relying upon other hydrogen utilizing anions for prevention of acid-base imbalance may be a feasible compromise formula.

FIGURE LEGENDS

Fig. 1 - Normal appearing arcuate nucleus of hypothalamus from a mouse pup given a high dose (100 µl/g) of the amino acid control solution 5 hours previously (X 130).

Fig. 2 - Arcuate nucleus of a mouse pup given a high dose (100 µl/g) of Aminosol^{R}. An inconspicuous (+) lesion affecting approximately 10 neurons at the angle of the 3rd ventricle (→ ←) is evident upon close inspection. Degenerating neurons have clear dilated cell bodies surrounding a shrunken black nucleus. Lesions of this size and appearance were characteristically seen in pups treated either with a high dose of Aminosol^{R} or with low doses of the casein preparations CPH^{R} and Travamin^{R} (X 130).

Fig. 3 - Arcuate nucleus of a mouse pup given a high dose (100 µl/g) of the casein hydrolysate Travamin^{R}. A large (+++++) lesion involving 40-50 arcuate neurons in various stages of degeneration is evident. Lesions of this size were seen in animals treated with high doses of either CPH^{R} or Travamin^{R} but not with Aminosol^{R} (X 130).

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TABLE 1 - FREQUENCY* AND SEVERITY** OF HYDROLYSATE-INDUCED BRAIN DAMAGE

Solution	Dose (µl/g) 20	40	60	80	100
Control	0	0	0	0	0
Aminosol^{R}	0	0	0	2(+)	3(+)
CPH^{R}	0	4(+)	6(++)	6(+++)	6(+++++)
Travamin^{R}	1(+)	4(+)	6(++)	6(+++)	6(++++)

*The number of animals (out of 6 treated with each solution at each dose) sustaining hypothalamic damage.

**Necrotic neurons per representative section (NN/sec) per animal averaged for affected animals of each group. + = 1 to 10 NN/sec; ++ = 11 to 20 NN/sec; +++ = 21 to 30 NN/sec; ++++ = 31 to 40 NN/sec; +++++ = 41 to 50 NN/sec.

Biochemical Changes of Brain and Liver in Neonatal Offspring of Rats Fed Monosodium-L-Glutamate

Much attention has recently been focused on some of the possible hazards of high levels of monosodium-L-glutamate (MSG) given by injection or fed to several species of experimental animals. These effects range from obesity and neuroendocrine disturbances to sterility and brain lesions[1-8]. In a previous study[9] we attempted to establish a biochemical basis for the action of MSG by feeding it to rats at levels up to 20% of the diet and then measuring the concentrations of a number of brain and liver constituents. Analysis of liver indicated that dietary MSG had no effect on protein, RNA, DNA, glutamate, lactate, malate or α-glycerophosphate. Concentrations of glutamate, glutamine, aspartate, DNA and protein and activity of glutamic decarboxylase (GAD) in brain remained constant while γ-aminobutyric acid (GABA) concentrations were significantly decreased in animals fed MSG. The rats ingesting MSG exhibited increased irritability, which may be related to decreased levels of brain GABA[9,10].

The purpose of the present investigation was to study the effects of dietary MSG on some selected brain and liver metabolites of second generation neonatal rats born to parents fed a diet supplemented with 10% MSG.

Materials and methods. Holtzman weanling rats were fed Purina laboratory chow alone or supplemented with 10% MSG for 100 days. The rats were mated on a one-to-

Table I. Body, brain and liver weights* of rats during postnatal development

Days	Control			Treated		
	Body weight	Brain weight	Liver weight	Body weight	Brain weight	Liver weight
1	7.21 ± 0.26	0.2871 ± 0.0058	0.280 ± 0.011	7.39 ± 0.20	0.2939 ± 0.0088	0.288 ± 0.009
2	8.40 ± 0.26	0.3400 ± 0.0063	0.336 ± 0.010	8.64 ± 0.27	0.3488 ± 0.0089	0.332 ± 0.009
3	10.32 ± 0.17	0.4148 ± 0.0068	0.410 ± 0.011	9.47 ± 0.23	0.4003 ± 0.0085	0.358 ± 0.018
5	13.13 ± 0.52	0.5442 ± 0.0082	0.485 ± 0.016	13.59 ± 0.57	0.5882 ± 0.0154	0.453 ± 0.019
10	23.55 ± 0.39	1.0132 ± 0.0117	0.796 ± 0.017	24.43 ± 0.38	1.0900 ± 0.0127	0.726 ± 0.020
21	51 ± 3	1.4242 ± 0.0243	1.934 ± 0.137	52 ± 1	1.4356 ± 0.0187	1.963 ± 0.061

* Each value is the average of 10 rats and is expressed as g ± S.E.M. The control rats were born of parents fed Purina chow. Treated rats were born of parents fed Purina chow supplemented with 10% MSG. All 20 control rats conceived and 17 of the 20 treated rats conceived; the average litter sizes were 9.7 ± 0.4 and 10.2 ± 0.5 pups/litter, respectively.

one basis for a 7-day period and the resultant offspring (first generation; F_1) after weaning were continued on the same diet as their respective parents for 100 days. At this time the F_1 generation rats were mated as described above and 10 neonatal offspring (second generation; F_2) from each group were sacrificed at days 1, 2, 3, 5, 10 and 21. Brains were removed for determinations of GAD, GABA, glutamate, aspartate, protein and DNA by previously described methods[11]. Livers were assayed for RNA, DNA, protein and glutamate. The stomach contents of the 5-day-old rats were also assayed for glutamate as an indicator of transfer of MSG by way of mothers' milk.

Mean values, standard errors and their significance were calculated according to the Student's *t*-test.

Results. No significant differences were noted in conception rate, pups per litter or body, brain and liver weights between offspring of control and MSG-fed rats (Table I) during the first 21 days of postnatal development. As seen in Figure 1, the brain-to-body weight ratio was significantly higher ($P < 0.05$) for treated rats at day 3, whereas the liver-to-body weight ratio was lower than that of controls at days 3 and 5 ($P < 0.05$). These differences disappeared before the neonatal rats were weaned.

Figure 2 shows that the protein content of the brains of treated rats parallels that of the controls. The DNA concentrations in brains of control and treated rats were remarkably similar (Figure 3) and, when expressed as

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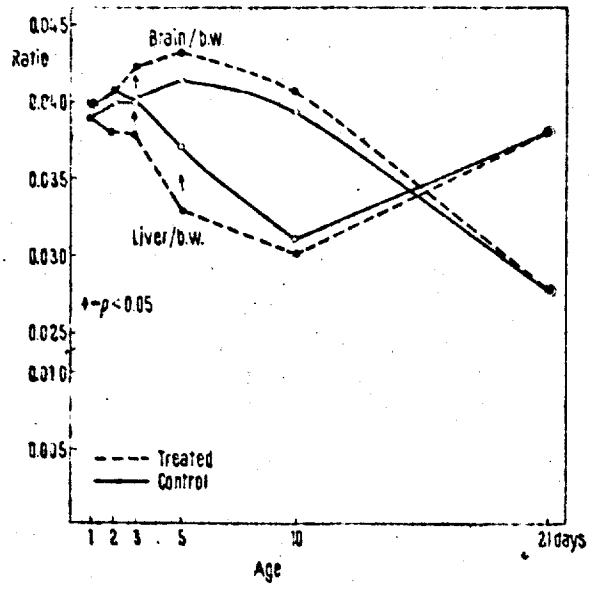

Fig. 1. Effect of 10% dietary MSG on brain-to-body weight and liver-to-body weight ratios of F_2 generation neonatal rats.

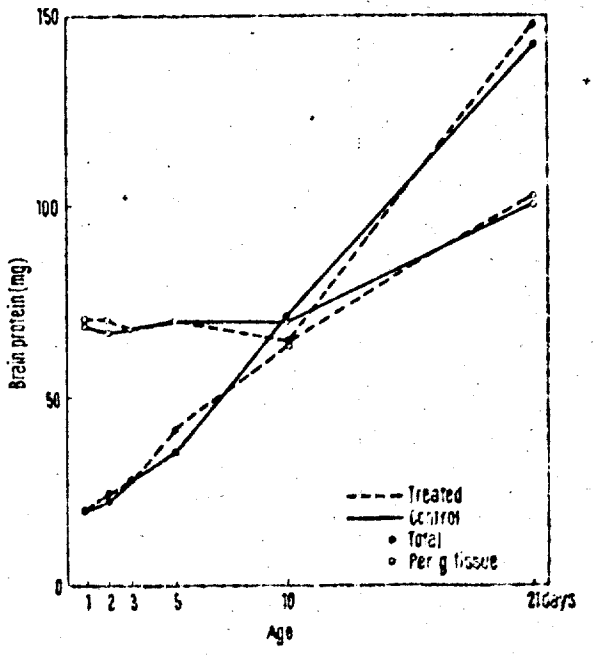

Fig. 2. Effect of 10% dietary MSG on brain protein of F_2 generation neonatal rats.

mg/g tissue, were comparable to adult values at day 5 of postnatal development. Figures 4 and 5 show the concentrations of aspartate, glutamate and GABA in brain. Aspartate and glutamate did not reflect the increased dietary intake of MSG; however, GABA was significantly elevated ($P<0.001$) on day 1. This elevation did not persist and by day 2 the values were down to normal.

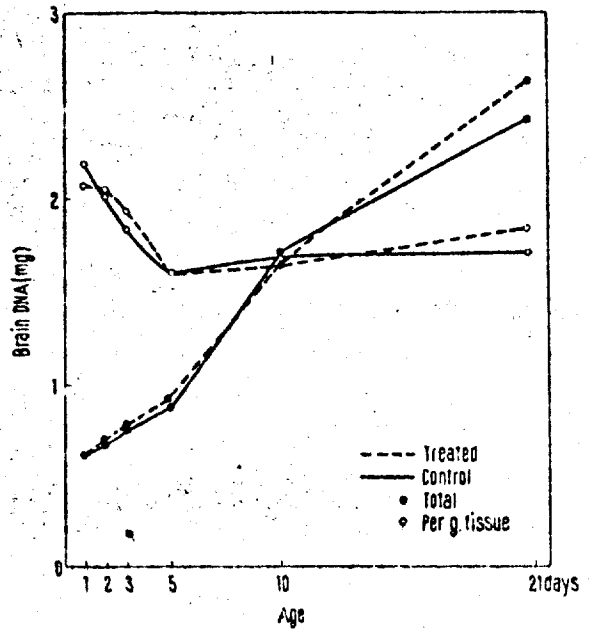

Fig. 3. Effect of 10% dietary MSG on brain DNA of F_3 generation neonatal rats.

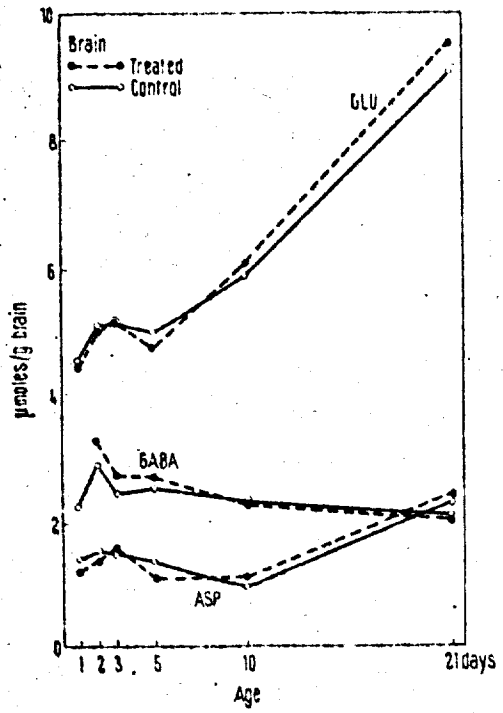

Fig. 4. Effect of 10% dietary MSG on concentrations of glutamate (GLU), aspartate (ASP) and γ-aminobutyric acid (GABA) in brains of F_3 generation neonatal rats.

GAD, the enzyme responsible for the formation of GABA, did not change significantly in treated rats; activity varied from 62 to 100 μmoles GABA formed/g protein/h during the 21-day postnatal period.

The results in Table II show that 10% MSG in the diet had no effect on liver protein, RNA-P, DNA-P or glutamate.

Examination of the stomach contents of 5-day-old rats showed that offspring of parents fed the MSG diet had 20% more free glutamate than controls (0.574 μmole as compared to 0.476 for controls; $P<0.05$). Treated offspring had rough, shaggy-hair coats which became normal during the third week.

Discussion. Feeding MSG to rats through several generations had no effect on reproductive function as measured by conception rate and pups born per litter. These data are in agreement with earlier reports from this agency[12] and other laboratories who studied the rat[9,13]. Furthermore, no differences were noted in the development of the rat during the postnatal period as measured by brain, liver and body weights. The increased brain-to-body weight ratio at day 3 and decreased liver-to-body weight ratios at days 3 and 5 for rats fed MSG were transient effects from which the animals recovered by day 10.

DNA concentrations in brain reflect cell number and size, and a decrease is associated with neuron deficiency[14]; the values found in this study were not different for control and MSG-fed rats. Protein concentrations in brain, as well as those of DNA, are indicative of growth and development of this organ and are markedly decreased in mal-

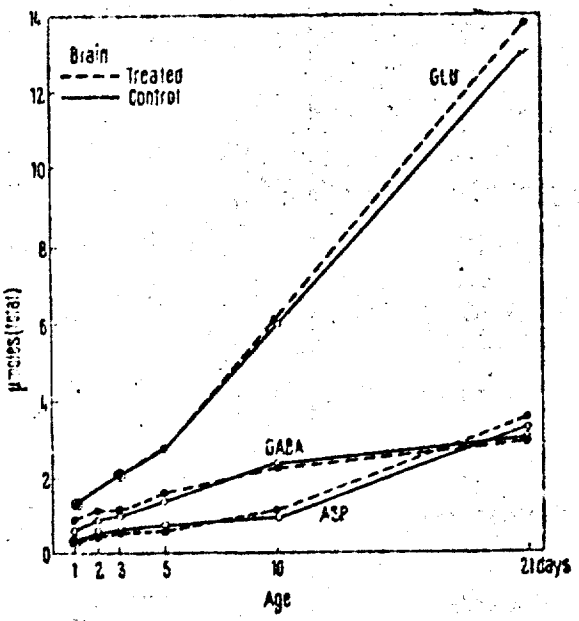

Fig. 5. Effect of 10% dietary MSG on total brain content of glutamate (GLU), aspartate (ASP) and γ-aminobutyric acid (GABA) in F_3 generation neonatal rats.

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Table II. Liver protein, RNA-P, DNA-P and glutamate* in rats during postnatal development

Days	Control				Treated			
	Protein	RNA-P	DNA-P	Glutamate	Protein	RNA-P	DNA-P	Glutamate
1	138 ± 8	0.913 ± 0.037	0.294 ± 0.029	4.27 ± 0.24	127 ± 2	0.956 ± 0.031	0.295 ± 0.017	3.46 ± 0.11
2	112 ± 2	0.965 ± 0.031	0.288 ± 0.017	4.41 ± 0.15	122 ± 7	0.899 ± 0.022	0.314 ± 0.025	4.31 ± 0.17
3	146 ± 12	1.072 ± 0.065	0.380 ± 0.014	4.21 ± 0.17	130 ± 9	1.107 ± 0.041	0.400 ± 0.010	4.05 ± 0.22
5	134 ± 12	1.035 ± 0.022	0.344 ± 0.009	3.57 ± 0.25	128 ± 3	1.067 ± 0.016	0.314 ± 0.009	4.28 ± 0.29
10	149 ± 5	0.942 ± 0.014	0.280 ± 0.011	3.22 ± 0.09	166 ± 12	0.962 ± 0.032	0.284 ± 0.008	3.43 ± 0.07
21	171 ± 8	0.930 ± 0.164	0.251 ± 0.036	4.09 ± 0.21	166 ± 5	0.954 ± 0.133	0.266 ± 0.029	4.51 ± 0.25

* Each value is the average of 5 rats and is expressed as mg/g liver ± S.E.M. for protein, RNA-P and DNA-P and as μmole/g liver for glutamate. Control rats were born of parents fed Purina chow. Treated rats were born of parents fed Purina chow supplemented with 10% MSG.

nutrition[15]; these concentrations were also unaffected by dietary MSG.

In a previous study[16], in which rats were fed MSG at levels up to 20% of the diet for 15 weeks, we found that GABA concentrations were reduced but GAD activity was not affected. In the present study, we found that mothers' milk from MSG-fed rats contained 20% more free glutamate than did controls, resulting in increased concentrations of GABA in brains of offspring at day 1. ADKINS et al.[17] reported a similar increase in free glutamate of milk from MSG-fed rats. The transient rise in GABA probably resulted in 'activation' of enzymes responsible for the metabolism of GABA and therefore the GABA concentrations were decreased to control values by day 2. Brain aspartate and glutamate were unresponsive to MSG in the diet.

Liver protein, RNA-P, DNA-P and glutamate levels were also independent of dietary MSG. The high activities of transaminases and oxidases which metabolize glutamate in liver are sufficient to maintain glutamate concentrations at 4 μmole/g liver. The activities of the corresponding enzymes in the brain will be reported in a future paper.

These generation studies are in general agreement with our earlier reports on effects of dietary level of MSG in that we were unable to find changes in biochemical components of brain and liver. We have now reached the F_4 generation with no further effects noted except for the rough, shaggy-hair coat which persists for approximately 30 days.

Zusammenfassung. Nachweis, dass die Ernährung von Ratten mit Monosodium L-Glutamat (MSG) im Laufe der ersten 21 Tage nach der Geburt keinen Einfluss auf die Entwicklung sowie auf das Körper-, Gehirn- und Lebergewicht hatte. Diätetisches MSG hatte ausserdem keinen Effekt auf Protein, Aspartat und Glutamat im Gehirn.

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[18] Acknowledgments. The authors would like to thank Mr. T. S. BOND and Mrs. R. M. BRANCH for their technical assistance and Mrs. H. ROGASSKI for her excellent photography.

Erythrocyte NADH-Methemoglobin Reductase Activity in Experimental Riboflavin Deficiency

Measurement of activity of enzymes requiring vitamin cofactors has been shown to be a sensitive and specific means of detecting vitamin deficiencies[1-3]. A flavin-dependent enzyme, NADH-dependent methemoglobin reductase, has been proposed as a possible indicator of the status of riboflavin nutrition[4]. Activity of the enzyme in erythrocytes from normal subjects is enhanced approximately two-fold by addition of flavin-adenine-dinucleotide (FAD) to the assay system[4], but activity of the enzyme from riboflavin-deficient subjects has not been studied. The purpose of this paper was to study erythrocyte NADH-methemoglobin reductase activity in riboflavin deficient rats, and to determine the effect of addition of FAD upon activity of the enzyme.

Materials and methods. 24 Sprague-Dawley weanling rats were divided into 4 groups. Groups 1 and 2 were fed a riboflavin-deficient diet (Nutritional Biochemicals). Groups 3 and 4 were given a regular balanced diet containing 794 mg of the vitamin per kg. Supplements of 80 μg of riboflavin were given by s.c. injection every other day to rats in Groups 2 and 3 in accordance with established riboflavin requirements[5]. 2 rats from each group were sacrificed on days 21, 23 and 26 following initiation of the diets. Blood was collected by cardiac puncture, and the liver was removed. Erythrocyte methemoglobin reductase activity was assayed by the method of HEGESH et al.[6] and

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PLACENTAL TRANSFER OF MONOSODIUM GLUTAMATE (MSG). Roy M. Pitkin, Ann Reynolds, Lloyd J. Filer, and L. Stegink, Univ. of Ia. Col. of Med., Depts. Ob-Gyn. and Peds., Iowa City, Ia. (Intr. by J.T. Bradbury).

L-glutamic acid-3, 4-^{14}C was administered to pregnant rhesus monkeys and serial maternal and fetal plasma samples were analyzed on an amino acid analyzer equipped for simultaneous radioactivity and amino acid measurement, permitting determination of amino acid composition, specific activity of glutamate, and isotope incorporation into metabolically derived compounds. In maternal plasma, 68% of radioactivity remained in association with glutamate, 22% was converted to a pre-taurine ninhydrin negative acidic compound, and small amounts were converted to a post-taurine ninhydrin negative acid, aspartate, glutamine, and ornithine. In the fetus, however, the two acidic compounds together accounted for more than 80% of the radioactivity and less than 2% of the counts represented glutamate. Chemically, although maternal plasma glutamate levels increased 25 fold, fetal levels remained constant.

The results indicate that the hemochorial placenta is virtually impermeable to L-glutamic acid as such and suggested that, either during the process of placental transfer or elsewhere in the maternal system, glutamate is metabolized to 1 or 2 acidic compounds which then traverse the placenta. The nature of these 2 compounds is under investigation; two likely possibilities are alpha keto glutaric acid and oxaloacetic acid.

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EFFECTS OF MONOSODIUM GLUTAMATE (MSG) ADMINISTRATION ON RATS DURING THE INTRAUTERIN LIFE AND THE NEONATAL PERIOD

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EFFECTS OF MONOSODIUM GLUTAMATE (MSG) ADMINISTRATION ON RATS DURING THE INTRAUTERIN LIFE AND THE NEONATAL PERIOD

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Considerable attention has been given in the last few years to the safety for use of MSG as a flavour enhancer in baby foods. The findings by OLNEY (1) and those by OLNEY & SHARPE (2, 3) — although controversial and not supported by OSER et al. (4), LOWE (5) and ZAVON (6) — have renewed interest for previous research (LUCAS et al. (7)), also reporting negative effects after coercitive administration of MSG to rodents. The alarm caused mainly to the public opinion has led some of the major U. S. manufactures to suspend the use of this ingredient in baby foods, awaiting a clarification of the controversy (1), (2), (3).

The development of retinal and brain lesions with relatively low loads of glutamate (1 g/kg b.w.) has been reported only in rodents during the neonatal period (i.e., at the phase preceeding the development and maturation of the hemato-cerebral and hemato-retinal barrier) (8). Similar, if not identical, retinal and brain lesions seem to be caused, however, also in adult mice, by injecting sublethal doses (6-8 g/kg b.w.) (1).

We have also to consider that a concentration gradient exists between foetal and maternal blood. In the case of glutamic acid, the gradient is higher than 2:1 (9), (10). This raises the problem of verifying whether a continuous administration of MSG during pregnancy represents an accumulative risk for the intra-uterine development especially.

One of the related controversial points is whether MSG is truly toxic, even if administered orally, as it has been found to be in the case of parenteral administration. In this context we have, therefore, deemed useful to study the effects of a daily administration of MSG

added, at different levels, to the diet in two successive generations of rats.

In the present paper, in addition to the effect on the cellularity of the central nervous system, we shall investigate the reproductive performance, the growth and the weight reached in adult life. The results of the histological studies of the brain will be the subject of another work.

Materials and Methods

The experiments have been carried out on rats of the Sprague-Dawley strain, bred by the Istituto Nazionale della Nutrizione, specifically in order to:

1) study the reproductive performance on 54 female rats with an average weight of 243 g (parental generation) and on 38 females with an average weight of 250 g (first generation);

2) examine the growth rate of each litter, from birth up to the 30th day of life; on 418 female and male rats of the first generation and on 270, males and females, of the second generation;

3) analyze the effects of MSG administration on the cellularity of the CNS in 115 rat brains of females and males of the first generation and in 108 brains of females and males of the second generation.

During the whole experimental period, the diets reported on table 1 were given ad libitum to the tested animals; it should be pointed out that, regarding the vitamin content, the effects of three different vitamin mixture levels were studied with respect to the response to MSG administration, i.e. 0.5%, 1%, 2% respectively. This experimental scheme was adopted after preliminary observations, carried out by accidentally administering an outdated vitamin mixture, had shown the possibility that tolerance to the addition of MSG be lower in the case of inadequate vitamin content in the diet. Each lot of animals at different vitamin levels was divided into three groups, the first of control, the second on 1% MSG and the third on 2% MSG. The addition of MSG has been carried out by replacement of an equal percentage of toasted starch.

Concerning the beginning of the experiment starting from the parental generation, pregnant females, on about the 5th day of pregnancy, were isolated in single cages, at a constant temperature

TABLE I. - *Experimental diet composition* (Ingredients %).

	Basal diet Vitamin mixture 0,5 %	Basal diet Vitamin mixture 1 %	Basal diet Vitamin mixture 2 %
Casein	21	21	21
Toasted rice starch . .	54,5	54	53
Sugar	10	10	10
Margarine	9	9	9
Cod-liver oil	1	1	1
Salt mixture	4	4	4

The MSG addition (1%, 2%) was made by removing a corresponding percent of toasted rice starch.

level, and fed for the whole period of pregnancy on the various experimental diets. Observed were the reproductive performance, the number of successful pregnancies, the size and growth of the litters which were reduced to the standard number of 8 on birth and of 6 on the 15th day, and on the survival rate. After weaning, the females were fed the same diet administered to their mothers until they reached sexual maturity.

Between the third and fifth month of age, these first generation females were mated to produce the second generation, following the method already mentioned.

Some of the litters of the first and second generation were sacrificed by decapitation: some at birth, others on the 15th and 30th day of age. The brains were thus isolated, analytically weighed and stored at —80°C before being homogenized with TCA 7%.

Following the methodology described by SMITH & THANNHAUSER (11), the homogenized material has been submitted to various washing procedures.

The determination of the RNA content was carried out on the combined supernatants, according to the methodology by SCHNEIDER (12).

After incubation at 90% and washing with PCA 1.5%, the DNA was determined on the (thus obtained) combined supernatants following the method described by BURTON (13).

Only one modification has been brought to the SMITH & THANNHAUSER method: all the washing procedures were made beforehand,

in order to eliminate from the homogenized material the sugars and fats which are interfering with the colorimetric determination of RNA.

Results and discussion

Daily consumption of food and MSG

Table 2 reports food consumption of each lot and related groups. It shows that food consumption is logically affected by the vitamin content of the diet. With regard to MSG addition, only in the parental generation at 0.5% vitamin and during the first 15 days post-partum can a significant difference be observed between the food consumption rate of the control group and that of the 2% MSG group. This seems to show that the MSG appetizing properties, at least in rats, are revealed only in particularly critical conditions, i.e. in connection with a sub-marginal level of vitamin content and with the lactation period.

Table 2 also shows data concerning the average consumption rate of MSG in the various experimental conditions. From the examination of the values expressed in g/kg body weight/day, it follows that at the two addition levels studied, consumption varies from the first to the second generation between 0.5-0.6 and 1.2-1.4 g/kg b.w. during pregnancy; between 0.7-0.8 and 1.4-1.8 g/kg b.w. during lactation period; and between 0.8-1.5 and 2.1-3.0 g/kg b.w. during the period including the beginning and the end of the weaning period.

These consumption levels, with regard to the influence of oral administration on the reproductive performance through successive generations are, therefore, partly comparable with those studied by Khera et al. (14).

Taking into account the cumulative response following a prolonged administration, these levels seem, moreover, particularly interesting when related to what observed by Olney (1), and Olney & Sharpe (2, 3) after a single subcutaneous or oral administration (intubation) of doses between 0.55 g/kg b.w.

Reproductive performance

Table 3 shows that in the parental generation the addition of MSG to the diet of each lot at different vitamin levels increases the percentage of successful pregnancies. In the first generation,

TABLE II. - *Daily consumption of diet.*

	During the pregnancy			1st - 15th day			16th - 30th day		
	Diet (g pro die)	MSG (mg tot. pro die)	MSG (mg/kg P.c. pro die)	Diet (g pro die)	MSG (mg tot. pro die)	MSG (mg/kg P.c. nurse — rat pro die)	Diet (g pro die)	MSG (mg tot. pro die)	MSG (mg. kg P.c. nurse + nest pro die)
MATERNAL GENERATION				Vitamines 0,5 %					
Control	13,2	0	0	14,3	0	0	28,0	0	0
MSG 1 %	15,8	158,0	592 3	16,2	162,0	691,1	24,4	244,0	846,6
MSG 2 %	14,8	296,0	1104,7	18,5*	310,0	1646,2	32,4	648,0	2491,3
				Vitamines 1 %					
Control	16,7	0	0	23,7	0	0	23,6	0	0
MSG 1 %	13,5	135,0	568,3	21,3	213,0	773,5	33,9	393,0	1235,2
MSG 2 %	16,6	332,0	1246,5	21 1	422,0	1496,1	42,1**	842,0	2777,1
				Vitamines 2 %					
Control	18,2	0	0	24,1	0	0	44,5	0	0
MSG 1 %	15,5	155,0	558 6	25,4	254,0	977,9	46,5	465,0	1466,3
MSG 2 %	18,1	362,0	1277,2	24,6	492,0	1779,3	48,3	966,0	3011,8
				Vitamines 1 %					
1st GENERATION				....			....		
Control	17.3	0	0	24,8	248,1	802,6	42 9	429,3	1184,2
MSG 1 %	17,5	174,8	528,1	23,4	468,0	1485,9	35,4	707,6	2143,2
MSG 2 %	15.4	308,6	1036,5						
				Vitamines 2 %					
Control	17,4	0	0	....			....		
MSG 1 %	15,8	157,7	626,9	23,2	239,6	966,2	33,3	332,9	1097 8
MSG 2 %	17,6	359,4	1422,5	23 5	470,2	1834,5	33,5	669,6	2097,1

* 0,05 > P < 0,01; ** significance limit.

where, however, the lot at 0.5% vitamin is missing, the addition of MSG increases the successful pregnancy rate from 80% to 100% when the vitamin content is at the optimum level (2%).

This is in clear contrast with the data furnished by KHERA et al. (9) who, with lower doses (expressed as g/b.w) have observed an apparently slight decrease in fertility. Apart from the interpretation of the responsible mechanisms, among those brought forth in order to explain the MSG effects on reproductive performance this result is undoubtedly of remarkable practical interest.

Furthermore, the subsequent test on the reproductive performance shows that, in agreement with the various unpublished reports submitted by the Food & Drug Administration to the Food Protection Committee in the U.S. (8), no differences exist between the various groups as far as the average size (number of born) and average weight per capita of litters on birth is concerned. The breeding percentage shows, on the other hand, remarkable results. In the first generation, the percentage of new-borns reaching weaning age, apart from the effect of vitamin content, tends to increase in connection with the addition of MSG. In the second generation we can also observe that, while no controls at the two vitamin levels survive weaning, the breed percentage is quite high in the MSG treated groups, particularly at the 2% vitamin level.

No significat differences were found, however, in the individual average weight at the weaning.

Growth and weight in adult life

That the addition of MSG to the diet has no influence on growth rate in neonatal period is further demonstrated, at least in the first generation, by data corresponding to the lactation value (*), provided by GOYCO & ASENJO (15). Apart from the possibility that the addition of MSG to maternal diet may or not increase the contents of MSG in the milk this shows that the MSG administration doesn't trouble the lactation whose efficiency regarding the growth is not altered.

The mid-term effects of MSG administration show another particularly interesting finding: the weight reached in adult life. Against

(*) LACTATION VALUE = (*increase in weight of standard litter*) - (*loss in weight of mother*) / grams of proteins used by mother.

TABLE III. - *Reproductive performance.*

	Pregnancy %			Mean size of nest at birth			Mean weight at birth			Breeding %			Mean weight at weaning		
	C	MSG 1	MSG 2	C	MSG 1	MSG 2	C	MSG 1	MSG 2	C	MSG 1	MSG 2	C	MSG 1	MSG 2
MATERNAL GENERATION															
Vitamins 0,5 %	66,6	83 3	83,3	9	9	9	5,45	5,86	5,88	21,0	16,7	40,7	56,70	78,50	60,70
Vitamins 1 %	83,3	83,3	100,0	9	8	9	5,60	5,64	5,93	74,3	70,6	80,9	93,02	102,90	89,90
Vitamins 2 %	83,3	100,0	100,0	9	9	10	5,74	5,53	5,70	75,0	92,7	90,3	111,90	97,00	98,58
Ist GENERATION															
Vitamins 1 %	80,0	85 7	71,4	8	8	7	6,10	5 84	5,60	0	56,1	36 1		76,00	79,50
Vitamins 2 %	80,0	100,0	100,0	8	7	8	5,63	5,87	6,11	0	87,2	83,9		70,14	74,75

TABLE IV. - *Lactation value* (11th generation).

	Vitamins 0,5 ‰	Vitamins 1 ‰	Vitamins 2 ‰
Control	2,59 ± 1,35 (4)	2,98 ± 1,83 (5)	2,98 ± 1,14 (5)
MSG 1 %	2,09 ± 1,02 (5)	2,81 ± 1,94 (5)	2,76 ± 1,11 (6)
MSG 2 %	2,44 ± 1,50 (5)	1,90 ± 1,17 (6)	2,79 ± 1,10 (6)

the results of OLNEY (1) and of OLNEY & SHARPE (2, 3), no female fed a higher MSG level have shown any sign of obesity.

Cellularity of NCS

According to ENESCO & LEBLOND (16) as well as WINICK (17) and WINICK & NOBLE (18), the study of the cellularity of both the whole organism and single organs may be based on the nucleic acids and protein content.

Assuming that DNA is mainly located in the nucleus of any kind of diploid cell, it is thus possible to estimate the total figure of the nuclei (in million) and the weight per nucleus (in mμg).

We can thus explore three developmental stages in every organism: hyperplasia, hyperplasia and hypertrophy and hypertrophy alone. The passage from one stage into the other depends, essentially, first on the decrease and then on the standstill of the DNA synthesis.

This may be observed on rat brains between the 17th and 21st day of life, while, as far as protein content is concerned, they may increase till the 89th day.

Obviously, the NCS cellularity values express mainly the increase of glial cells and microneurons (19). Independently from the circumscribed cerebral lesions observed by OLNEY (1) and OLNEY & SHARPE (2, 3), the study of cellularity allows us to evaluate the overall effects on brain development following a constant administration of MSG.

Considering the higher daily dose levels adopted in our test and the fact that, according to MCLAUGHLAN et al. (20), the glutamic acid concentration in the brain ranges more than 100 times above that

TABLE V. - *Nervous central sistem cellularity* (1st generation).

Vitamins 0,5 %

	RNA mg/g tissue	DNA mg/g tissue	Protein mg/g tissue	Nucleus number (millions)	Cellular size (nucleus weight) mμg	RNA / nucleus mμg	Protein / cell mμg
AT BIRTH							
Control	3,15 ± 0,84	2,67 ± 0 76	32,30 ± 10,19	90,46	2,49	7,55	75,2
MSG 1 %	3,30 ± 0,80	2,67 ± 0,42	30,23 ± 5 82	100,06	2,35	7,38	70,8
MSG 2 %	3,37 ± 0,48	2,48 ± 0,18	32,55 ± 0,53	97,47	2,50	8,36	80,6
AT 15th DAY							
Control	2,77 ± 0,01	1,30 ± 0,13	58,47 ± 6,43	243,32	4,75	13,22	278,8
MSG 1 %	2,90 ± 0,72	1,33 ± 0,20	67,21 ± 25,26	240 73	4,71	13,42	320 1
MSG 2 %	3,62 ± 0,78	1,42 ± 0,14	66,25 ± 21,31	264,39	4,41	15,73	269,0
AT WEANING							
Control	3,24	1,43	76,43	346,73	4,34	14,09	332,2
MSG 1 %	3,05 ± 0,45	1,54 ± 0,09	70,71 ± 8,14	375,14	4,02	12,09	283,5
MSG 2 %	3 15 ± 0,29	1,52	74,92 ± 3,94	358,08	4,08	12,84	305,5

TABLE VI a - *Nervous central sistem cellularity* (1st generation).
Vitamins 1 %

	RNA mg/g tissue	DNA mg/g tissue	Protein mg/g tissue	Nucleus number (millions)	Cellular size (nucleus weight) mμg	RNA / nucleus mμg	Protein / cell mμg
AT BIRTH							
Control	4,06 ± 0,35	2,96 ± 0,39	28,45 ± 2,10	102,77	2,11	8,27	80,4
MSG 1 %	2,37 ± 0,25*	2,11 ± 0,15**	30,78 ± 2,64***	81,44	3,09	7,36	96,0
MSG 2 %	3,08 ± 0,39***	2,22 ± 0,39**	25,88 ± 3,93*	82,99	3,00	8,69	72,3
AT 15th DAY							
Control	2,75 ± 0,46	1,31 ± 0,29	53,50 ± 5,00	247,86	4,93	13,23	259,7
MSG 1 %	3,04 ± 0,53	1,45 ± 0,40	56,54 ± 7,60	271,69	4,55	13,57	254,7
MSG 2 %	3,35 ± 1,18	1,23 ± 0,27	50,80 ± 6,59	221,05	5,21	16,61	260,5
AT WEANING							
Control	2,86 ± 0,22	1,36 ± 0,16	64,00 ± 1,41	331,94	4,62	13,21	296,0
MSG 1 %	2,86 ± 0,26	1,54 ± 0,10	71,92 ± 12,36	397,87	4,02	11,51	288,4
MSG 2 %	2,64 ± 0,24	1,34 ± 0,12	62,44 ± 4,18	348,58	4,63	12,17	288,9

* $P < 0,001$; ** $P < 0,05$; *** $P < 0,01$

TABLE VI b - *Nervous central sistem cellularity* (2nd generation).
Vitamins 1 %

	RNA mg/g tissue	DNA mg/g tissue	Protein mg/g tissue	Nucleus number (millions)	Cellular size (nucleus weight) mμg	RNA / nucleus mμg	Protein / cell mμg
AT BIRTH							
Control	4 27 ± 0,11	2,60 ± 0,16	42,33 ± 6,37	97,91	2,39	10,22	101,5
MSG 1 %	4,48 ± 0,48***	2,78 ± 0,40**	35,92 ± 4,60 •	101,50**	2,28**	9,74*	80,8
MSG 2 %	4,19 ± 0,23***	2 69 ± 0,17**	31 30 ± 2,87*	104,24***	2,31**	9,69*	72,3
AT 15th DAY							
Control	....	....	....	....	....	....	....
MSG 1 %	3,58 ± 0,26	1,49 ± 0,07	37,00 ± 5,15**	269,46	4,16	14,85	153,8
MSG 2 %	2,62 ± 0 89	1.16 ± 0,30	31,03 ± 7,55*	190,69	5,62	13,78	167,7
AT WEANING							
Control	....	.....	....	....	....	....	....
MSG 1 %	2,10 ± 0,49	1,17 ± 0,29	30,44 ± 5,32**	218,17*	5,54	11,21	163,5**
MSG 2 %	2,07 ± 0,56	1,34 ± 0,33	33 73 ± 7,34***	275.10	5,22	10,43	172,2**

The values of significance here reported are referred to the corrisponding groups of the 1st generation.
*** $P < 0,001$; ** $P < 0,01$; * $P < 0,05$; • significance limit.

TABLE VII a - *Nervous central sistem cellularity* (1st generation).
Vitamins 2 %

	RNA mg/g tissue	DNA mg/g tissue	Protein mg/g tissue	Nucleus number (millions)	Cellular size (nucleus weight) mμg	RNA mμg / nucleus	Protein mμg / cell
AT BIRTH							
Control	4,07 ± 0,66	2,95 ± 0,36	27,37 ± 5,48	116,5	2,11	7,59	58,3
MSG 1 %	3,47 ± 0,45	2,83 ± 0,40	28,60 ± 6,68	99,24	2,22	7,70	62,7
MSG 2 %	3,19 ± 0,44**	2,77 ± 0,45	27,70 ± 5,01	92,93	2,30	7,21	63,3
AT 15th DAY							
Control	2,47 ± 0,48	1,55 ± 0,15	50 38 ± 7,99	301 85	4,01	9,83	200,2
MSG 1 %	3,33 ± 0 89	1,52 ± 0 16	57,77 ± 12,89	289,14	4,07	13,1	233,5
MSG 2 %	3,16 ± 0,30**	1,44 ± 0,08	51,32 ± 7,11	269,97	4,30	14,34	205,7
AT WEANING							
Control	2,77 ± 0,37	1,26 ± 2,55	65,45 ± 16,49	323,23	5,08	13,91	320,7
MSG 1 %	2,57 ± 0,26	1,38 ± 0,17	60,90 ± 9,97	342,45	4,54	11,77	288,3
MSG 2 %	3,18 ± 0,53	1,33 ± 0,17	66,11 ± 11,36	325,40	4,76	15,10	309,8

** $P < 0.05$

TABLE VII b - *Nervous central sistem cellularity* (2nd generation).

Vitamins 2 %

	RNA mg/g tissue	DNA mg/g tissue	Protein mg/g tissue	Nucleus number (millions)	Cellular size (nucleus weight) mμg	RNA / nucleus mμg	Protein / cell mμg
AT BIRTH							
Control	4,29 ± 0,16	2,63 ± 0,38	36,26 ± 7,80	98,97	2,30	10,26	86,3
MSG 1 %	4,14 ± 0,45**	2,80 ± 0,24	36,51 ± 3,71**	104,08	2,23 °	9,17*	81,1
MSG 2 %	4,17 ± 0,32***	2,85 ± 0,32	35,31 ± 5,67**	107,52	2,20 °	9,15***	77,3
AT 15th DAY							
Control	....	....	....	....	....	....	....
MSG 1 %	3,17 ± 0,50	1,32 ± 0,15 °	37,41 ± 3,19**	229,80	4,75 °	15,03	177,6
MSG 2 %	3,80 ± 1,70	1,42 ± 0,60	37,24 ± 18,02*	201,22*	4,85	16,48	161,2
AT WEANING							
Control	....	....	....	....	....	....	....
MSG 1 %	2,71 ± 0,50	1,35 ± 0,28	38,71 ± 4,67***	293,71	5,07	13,46	194,2***
MSG 2 %	2,13 ± 1,14	1,06 ± 0,43	29,55 ± 8,34***	231,21	6,92	11,92	186,2**

*** $P < 0,001$; ** $P < 0,01$; * $P < 0,05$; ° significance limit.

of the plasma level, the data shown in tables 5, 6a and 6b, and 7a and 7b are of particular interest.

The lot at 0.5% vitamins (table 5), probably due to a lower basic growth, show no statistically significant differences in the range of each parameter applied. On the other hand, in the lot at 1% vitamins in the first generation, some differences at birth are remarcable between the control group and groups at 1% and 2% MSG.

The last two groups show in fact significantly lower values of DNA, RNA and protein content. This means that, as a consequence of a reduced multiplication, the total figure of nuclei is smaller and the cell dimensions are larger in rats from mothers which have been fed a high MSG diet. The observed decrease at birth of DNA content seems to be in contrast with the unpublished results of the latest research by the Food & Drug Administration (8).

It is anyhow worth mentioning that between birth and the 15th day of life and beyond, these observed differences disappear as confirmed by the examination after weaning.

In the second generation (table 6 b), no differences are significant at birth. This seems clearly due to the fact that the second generation rats, coming from mothers trated with MSG since intrauterine life, show significantly higher DNA, RNA contents and brain nuclei than those of the first generation. The cell dimensions and consequently the proteins per cell seem, on the contrary, relevantly smaller than those of the first generation, especially on the 15th and 30th day of life. This effect, confirmed in the 2% vitamins lot, might indicate that the negative effect on N.C.S. cell multiplication observed at birth in the first generation (which is probably due to a deficiency in the enzymes level) which controls the metabolism of the glutamic acid (21, 22) is balanced by an adaptive mechanism.

Attention should also be given to the fact that in the second generation and the 2% vitamins lot, a statistically significant difference in protein content is observed after weaning between animals treated with 1% MSG and those with 2% MSG.

Although many aspects still need clarification concerning both controversial results and mechanisms, the results of our study seem to support the opinion expressed recently by RUBINI (23) that, contrary to the effects following parenteral administration, no evidence exists supporting toxicity after administration of MSG in the diet.

This is supported first of all, by improvement in the reproduc-

tive performance as well as by the absence of obesity in first generation rats, fed on diets rich in MSG since intrauterine life.

In addition, the reduced hyperplasia of NCS at birth in the first generation cannot be considered a negative element. In fact, during the neonatal period a complete recovery from hyperplasia and cell hypertrophy is evidenced. For all parameters, in fact, the data reported fall within the values considered as normal in the existing literature.

Finally, the fact that in the second generation the differences met in NCS cellularity at birth disappear, leads one to think that enzyme adaptive mechanisms play a part in it, normalizing, at least within certain limits, the metabolism due to MSG overdoses.

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SUMMARY

Different levels of MSG in conjunction with vitamin levels been studied in successive generations of rats.

At each vitamin level (0,5%, 1%, 2%) the rats received diets which contained MSG 1%, MSG 2%, no MSG (control groups).

Nests of the 1st and 2nd generation were studied for RNA, DNA, protein content, and the cellularity in the brain.

The MSG addition at both levels increases the number of accomplished pregnancies and determines a higher survival rate at weaning in the 2nd generation.

Rats born from mother which received diets with MSG during pregnancy have at birth a smaller number of nuclei and a bigger cellular size.

The RNA, DNA contents and nucleus number in the brain of rats born from mothers, which during their foetal life received MSG, are higher than in rats of the 1st generation.

The cellularity differences present at birth disappear at weaning in rats of the 2nd generation. This may suggest that enzymatic adaptation mechanisms have, within certain limits, counterbalanced the effects of high MSG dosage.

RIASSUNTO

In questo lavoro sono stati studiati gli effetti della assunzione di MSG con la dieta su due successive generazioni di ratti, in associazione a tre diversi livelli di vitamine.

Per ciascun livello vitaminico (0,5%, 1%, 2%) i ratti sono stati distinti in tre gruppi: il primo di controllo, il secondo all'1% di MSG, il terzo al 2% di MSG.

Su parte delle nidiate di prima e di seconda generazione è stato studiato il contenuto di RNA, DNA, proteine e la cellularità del Sistema Nervoso Centrale.

E' stato osservato che l'addizione di MSG aumenta la percentuale di gestazioni a termine e, in seconda generazione, la percentuale di soppravvivenza allo svezzamento.

A livello del Sistema Nervoso Centrale il numero dei nuclei è inferiore e le dimensioni cellulari sono maggiori nei ratti le cui madri hanno ricevuto durante la gestazione diete addizionate con MSG.

I ratti provenienti da madri che fin dalla vita intrauterina sono state trattate con MSG presentano un contenuto in DNA, RNA e numero di nuclei del cervello superiore a quello dei ratti della prima generazione.

La scomparsa in seconda generazione delle differenze riscontrate nella cellularità del Sistema Nervoso Centrale alla nascita fa pensare infine che meccanismi di adattamento enzimatico possano entrare in gioco normalizzando, entro certi limiti, il metabolismo dell'MSG ingerito in eccesso.

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Monosodium Glutamate: Effect on Plasma and Breast Milk Amino Acid Levels in Lactating Women[1] (36563)

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In 1957, Lucas and Newhouse (1) demonstrated that suckling mice injected with monosodium glutamate (MSG) at 2.2 g/kg body weight daily for 14 days developed retinal lesions. Since that time a number of investigators have confirmed the findings that suckling mice and rats injected with MSG or aspartate at these levels develop acute and irreversible retinal lesions (2–6). Adult mice were much more resistant to glutamate than newborn animals, and glutamate injection of pregnant mice produced no observable abnormalities in the offspring (1). Olney (7) and Olney and Sharpe (8) have reported that the arcuate nucleus of the hypothalamic region is particularly vulnerable to damage in the infant mouse, rat, rabbit and a single immature rhesus monkey when injected subcutaneously with MSG at doses ranging from 0.5 to 2.7 g/kg body weight. They have recently reported development of such lesions in infant mice after oral ingestion of 3 g/kg body weight of MSG, aspartate or cysteine (9). Although Arees and Mayer (10) reported the development of such lesions in adult rats when injected with large doses of MSG, several other groups of investigators have failed to induce CNS lesions in the newborn rat, monkey or dog with MSG (11–14).

Because questions have been raised on the basis of some animal studies about the safety of ingestion of MSG by lactating women, a series of MSG loading tests were carried out. This report describes the effect of the oral administration of MSG on free amino acid levels in plasma and breast milk.

Methods and Materials. A total of 10 women with well-established lactation patterns of 30 to 90 days duration were studied.[2] After an overnight fast the subjects received a 6 g load of MSG or lactose at 0800 hr contained in twelve 0.5 g capsules. This approximates a dose equal to 0.1 g/kg body weight, and represents considerably more than would be ingested with a meal. It is also a quantity sufficient to cause the symptoms of the Chinese restaurant syndrome in susceptible subjects (20–27). For nine tests the MSG was given in conjunction with 240 ml of Carnation Slender, for four tests MSG was given in conjunction with water. Four control subjects were given 6 g lactose in conjunction with water (Table I).

Milk samples were obtained at zero time, and at 1, 2, 3, 4, 6 and 12 hr after administration of MSG or lactose. Timed urine samples were obtained from each woman for the following intervals: 0800 to 1100 hr, 1100 to 1400 hr, and 1400 to 2000 hr. We had previously observed in animal studies that MSG was absorbed at differing rates depending on whether it was administered in conjunction with water or with food (15). Thus, blood samples were drawn at: 0, 30, 60, 120, and 180 min after administration of the MSG load with water, 0, 60, 90, 150, and 210 min after administration of MSG with Slender, and 0, 30, 60, 90, 150, and 210 min after administration of the lactose placebo.

The plasma was separated immediately from the blood samples, deproteinized with solid sulfosalicylic acid (16), and either an-

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TABLE I. Schedule of Participation in Loading Tests.*

Subject	Days of lactation		
	30	60	90
1	B	A	B
2	A	B	B
3	B		B
4	B		
5			A
6	B	A	B
7	C		
8		C	
9		C	
10			C

* A, MSG in water; B, MSG in Slender; C, lactose in water.

alyzed immediately or stored at —70° until analyses could be performed. Urine samples were immediately deproteinized with sulfosalicylic acid (16) and stored at —70° until analyzed. Milk samples were deproteinized by the addition of 50 mg solid sulfosalicylic acid/ml of milk. The sample was centrifuged, and the clear solution between the upper fat layer and the precipitated protein was carefully removed. If the solution was cloudy, it was centrifuged at 20,000*g* for 20 min to clear the solution prior to analysis or storage at —70°.

Amino acid analyses were carried out with Technicon NC-1 single column amino acid analyzers using the buffer system described by Efron (16) for physiological fluids.

Results and Discussion. Because experiments in this laboratory with newborn pigs and monkeys had demonstrated that animals given oral loads of 3,4-^{14}C-monosodium glutamate, in conjunction with water or an infant formula, incorporated significant quantities of radioactivity only into aspartate, glutamine, glutamate and alanine (15), the data in Tables II and III are limited to these four amino acids.

The changes noted in plasma amino acid levels in these women following the ingestion of MSG load are similar to those observed in the newborn pig or monkey given an oral dose of MSG (0.1 g/kg of body wt).

Evaluation of the plasma amino acid data in Table II indicates that plasma glutamate and aspartate levels peak approximately 30 min following ingestion of MSG with water in

TABLE II. Plasma Amino Acid Levels.

Time (min)	Amino acid (μmoles/100 ml plasma)			
	Aspartate	Glutamine	Glutamate	Alanine
MSG with water ($N = 4$)				
0	0.32 ± 0.16	61.9 ± 16.8	3.90 ± 1.70	53.1 ± 13.3
30	1.04 ± 0.92	68.0 ± 7.10	13.0 ± 10.1	50.6 ± 11.4
60	0.54 ± 0.31	62.4 ± 5.80	7.5 ± 4.80	46.6 ± 5.60
120	0.70 ± 0.40	65.9 ± 14.9	5.10 ± 2.70	47.3 ± 10.5
180	0.45 ± 0.25	62.4 ± 8.07	4.75 ± 2.31	40.5 ± 10.5
MSG with Slender ($N = 9$)				
0	0.64 ± 0.27	61.0 ± 3.70	4.34 ± 0.70	42.5 ± 6.32
60	1.28 ± 1.42	70.5 ± 14.7	7.05 ± 2.70	68.1 ± 19.6
90	1.27 ± 1.24	72.1 ± 14.0	9.23 ± 5.34	67.3 ± 12.1
150	1.84 ± 1.45	65.0 ± 10.4	11.8 ± 8.20	50.3 ± 10.4
210	1.32 ± 0.80	59.6 ± 8.31	10.2 ± 7.99	46.8 ± 6.95
Lactose with water ($N = 4$)				
0	0.43 ± 0.14	69.7 ± 16.6	4.00 ± 0.97	40.5 ± 6.60
30	0.65 ± 0.07	83.6 ± 8.70	4.10 ± 0.28	48.3 ± 6.70
60	0.39 ± 0.10	76.4 ± 4.60	3.70 ± 1.40	46.3 ± 10.0
90	0.56 ± 0.26	65.0 ± 16.6	3.80 ± 1.90	41.7 ± 11.4
150	0.61 ± 0.26	71.2 ± 10.9	5.70 ± 4.40	35.1 ± 3.50
210	0.26 ± 0.10	63.0 ± 14.1	3.20 ± 0.56	35.9 ± 4.00

TABLE III. Free Amino Acid Levels in Breast Milk Following Administration of MSG or Lactose.

Amino acid	Time following administration (hr)						
	0	1	2	3	4	6	12
				(μmoles/100 ml milk)			
				MSG with water ($N = 4$)			
Aspartate	2.9 ± 1.0	5.0 ± 3.0	8.0 ± 3.4	8.1 ± 3.9	7.9 ± 2.5	7.5 ± 3.1	9.1 ± 2.1
Glutamine	34.3 ± 19.1	35.8 ± 19.8	55.1 ± 20.3	52.5 ± 18.0	53.9 ± 7.70	51.0 ± 11.9	47.3 ± 12.7
Glutamate	107 ± 61.0	113 ± 27.3	126 ± 17.1	153 ± 66.7	145 ± 16.4	157 ± 36.0	181 ± 32.0
Alanine	16.5 ± 6.3	22.4 ± 6.8	30.1 ± 6.6	29.8 ± 7.5	31.0 ± 5.6	35.0 ± 6.1	35.0 ± 3.7
				MSG with Slender ($N = 9$)			
Aspartate	2.5 ± 1.3	2.9 ± 1.6	5.3 ± 1.7	7.9 ± 2.7	8.0 ± 4.3	10.3 ± 1.5	5.8 ± 2.9
Glutamine	51.8 ± 27.2	42.4 ± 16.2	44.5 ± 12.7	57.0 ± 19.8	53.4 ± 22.4	88.1 ± 27.5	57.9 ± 23.0
Glutamate	146 ± 51.1	118 ± 39.7	128 ± 50.4	150 ± 34.1	161 ± 53.8	182 ± 28.0	159 ± 32.3
Alanine	16.0 ± 6.0	16.4 ± 4.6	25.8 ± 9.1	29.6 ± 9.8	27.4 ± 10.3	33.0 ± 7.8	29.2 ± 10.8
				Lactose with water ($N = 4$)			
Aspartate	2.60 ± 1.6	3.0 ± 0.5	4.0 ± 1.6	4.3 ± 0.8	4.4 ± 4.5	4.2 ± 2.0	4.5 ± 2.0
Glutamine	79.5 ± 44.5	79.9 ± 55.1	68.7 ± 35.5	62.6 ± 34.0	67.3 ± 37.2	70.6 ± 38.0	85.1 ± 34.1
Glutamate	128 ± 36.6	147 ± 27.1	145 ± 16.2	167 ± 51.0	175 ± 49.2	158.7 ± 89.5	161 ± 34.1
Alanine	17.5 ± 7.4	22.7 ± 6.1	23.4 ± 5.2	23.4 ± 5.3	20.9 ± 10.9	26.1 ± 12.9	33.1 ± 10.4

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Carbohydrate Metabolism and Physical Activity in Rats Fed Diets Containing Purified Casein Versus a Mixture of Amino Acids Simulating Casein [1]

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ABSTRACT The effects of varying energy intake levels on physical activity and glucose metabolism were studied in young growing rats. Male rats 28 days of age were fed for 31 days diets containing either casein or an amino acid mixture simulating casein at 2 levels of calorie intake. Physical activity was measured and ^{14}C recovery from injected glucose-1-^{14}C, -6-^{14}C, and -U-^{14}C was determined as percentage of dose in expired CO_2, feces, and urine, from one to 24 hours after giving each rat his daily ration. The average revolutions per day run was higher for calorie-restricted animals, but there was no significant difference in activity due to substituting the mixture of amino acids for casein, although casein-fed rats tended to be more active. This increase in physical activity caused by caloric restriction was due to a significant difference in daytime activity, as all rats were equally active in the dark. There were no significant differences due to calorie level or nitrogen source in $^{14}CO_2$ recovery from glucose-6-^{14}C and glucose-U-^{14}C, but amino acid-fed rats converted more glucose-1-^{14}C to $^{14}CO_2$ as measured cumulatively 6 and 23 hours after injection. Calorie-restricted rats expired less glucose-1-^{14}C as $^{14}CO_2$ during the first 6 hours after injection, but this effect was not evident after 23 hours. There was a trend toward lower ^{14}C recovery from glucose-U-^{14}C in urinary citrate of amino acid-fed rats, although urinary citrate excretion was unaltered by diet. These data indicate a greater utilization of alternative pathways to the glycolytic scheme and tricarboxylic acid cycle for metabolism of glucose in amino acid-fed rats and several possible explanations are discussed.

Work reported from this laboratory in a previous paper (1) showed that for young rats fed 2 levels of nitrogen and at 2 levels of calorie intake, there were higher nitrogen gains when rats in the high calorie group received the nitrogen as casein rather than as a mixture of amino acids simulating casein. Rats of the same age fed diets providing similar nitrogen intakes at a lower calorie level showed no significant advantage in nitrogen storage for casein over amino acids. The dependence of nitrogen storage on calorie intake raised a question as to the effect the substitution of casein for amino acids might have on energy metabolism.

Caloric intake level has been reported to affect the physical activity of the rat, but the nature of this effect is uncertain. Hughes (2) reported increased physical activity in food-deprived animals, but Fabry et al. (3) interpreted their oxygen-consumption data as indicating reduced activity in animals that were intermittently starved. Caloric restriction has been implicated in the activity of pathways of carbohydrate metabolism. Lee and Lucia (4) reported a decrease in the direct oxidation of glucose with caloric restriction to the extent that severe restriction caused the hexose monophosphate pathway to disappear irreversibly. Benevenga et al. (5) noted that a starvation-refeeding regimen produced significant increases in pentose phosphate-metabolizing enzyme activity.

The present paper reports the effect in rats of substituting casein for a mixture of amino acids simulating casein as measured by ^{14}C-labeled glucose metabolism at either of 2 calorie intake levels. To study the effect, if any, of the dietary regimens on voluntary activity the rats were allowed access to activity cages.

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EXPERIMENTAL

Specific-pathogen-free male rats[2] obtained at 21 days of age were housed individually and fed a stock diet[3] for one week before being fed their particular experimental regimen. A control group of 6 rats was killed to determine initial carcass content of calories and nitrogen and to furnish data for correcting initial weight of the experimental animals to the ingesta-free basis. On the basis of these data, initial live weight was multiplied by 91.9% to obtain ingesta-free carcass weight. Contents of the gastrointestinal tract were removed and the carcasses homogenized in a Waring Blendor before analysis (6).

Four diets were used in this study: 1) high calorie level with casein as the nitrogen source; 2) high calorie level with an L-amino acid mixture simulating casein as the nitrogen source; 3) restricted calories with nitrogen provided as casein; and 4) restricted calories with nitrogen provided as amino acids. Diets at the high calorie level contained 3% nitrogen. Restricted-calorie diets were formulated with increases in proportion of protein or amino acids, vitamins, salts, and roughage so that, when calorie intake was reduced from the high calorie level by approximately one-third, intakes of those nutrients were the same as the intakes at the higher calorie level. The diets and care of the animals have been described earlier (1).

The casein and amino acids were assayed for purity as reported earlier (1). The rats, 6 per group, were housed in a well-ventilated room at 28°, 50% relative humidity, and 12 hours per day of darkness and light. After being fed the experimental diets for 2 weeks all rats were assigned at random to rotating-treadmill activity cages for 3 weeks and reading of revolutions turned were taken at 12-hour intervals coinciding with the darkness/light changeover. During this 3-week period 1-μCi doses of high specific activity glucose-1-^{14}C, glucose-6-^{14}C, and glucose-U-^{14}C were injected intraperitoneally at weekly intervals into each rat in a pattern of reversal (i.e., 2 rats in each group received glucose-1-^{14}C the first week, 2 rats received glucose-1-^{14}C the second week, etc.). Each 1-μCi dose involved the injection of 0.036 to 0.060 mg of glucose. All rats were placed in the respiration apparatus with their daily ration one hour before ^{14}C injection and remained there for 23 hours after injection where all ^{14}C lost in CO_2, feces, and urine was recovered. All solid samples were dried, ignited in an O_2 bomb, and $^{14}CO_2$ was trapped and counted as $Ba^{14}CO_3$. Respired $^{14}CO_2$ was collected at 30-minute intervals for the first 6 hours and again determined at 11 and 23 hours. All samples were counted at infinite thickness in a thin-window gas-flow counter with an anticoincidence correction.[4] Total CO_2 recovery was determined and all specific activities were converted to total activity and expressed as percentage of dose injected as suggested by Wang (7). This avoids the dilution effects of endogenous materials on specific activity pointed out by Wood (8).

Aliquots of the ^{14}C-labeled glucose samples used were ignited in an O_2 bomb and $Ba^{14}CO_3$ was recovered and counted. The counts recovered in 1 μCi were 92% of the theoretical amount (i.e., 2,042,810 dpm vs 2,220,000 dpm), close enough so that the difference could be explained by small errors in measuring initial volume, determining counter efficiency,[5] converting infinite to zero thickness values, etc. Eight of the experimental animals were killed within minutes of removal from the respiration apparatus. In these 8 animals the carcass ^{14}C as actually determined was 27.9% ± 3.9[6] of the injected dose, whereas the ^{14}C recovered in respiratory CO_2, feces, and urine was 68.4% ± 5.4 of the total indicating that about 96% of injected ^{14}C could be accounted for by actual measurement.

Urine was fractionated into a variety of components prior to counting for ^{14}C. Ether and chloroform extracts were made to determine radioactivity present as lactic acid or ketone bodies. Urea CO_2 was released and ^{14}C was counted following treatment of the urine with urease (9). Oxalate was precipitated as calcium oxalate (10) and sugars were precipitated as the osazones (11). Amino acids were absorbed and separated into acidic, neutral, and basic frac-

[2] Lew strain from Microbiological Associates, Bethesda, Maryland.
[3] D & G Research Animal Laboratory Diet, Price-Wilhoite Company, Frederick, Maryland.
[4] All samples were counted for long periods of time on a Sharp "Wide-beta" gas-flow counter having a low background.
[5] See footnote 4.
[6] Standard error of the mean.

tions by the use of Amberlite IR-4 (12) following hydrolysis of the urine with HCl. Creatinine was isolated by the method of Owen et al. (13). Non-amino organic acids were determined with paper chromatography after first passing the sample through a Dowex 50 column to remove amino acids and salts. The strips were developed in a 4:1:5 (v/v) upper phase N-butanol:acetic acid: water solvent system and were sprayed with 0.04% bromophenol blue in 95% ethanol. Spots were identified from standards, cut from the paper, ignited, and $Ba^{14}CO_3$ was determined. The amount of citric acid present was determined by titration of the eluted material with a dilute base.

This study was conducted according to a randomized complete block design where blocks were composed of those 4 animals, one per group, which received the 3 labeled glucose moieties in the same order of injection. Analyses of variance were conducted treating the investigation as a 2 × 2 factorial.

RESULTS AND DISCUSSION

Physical activity. The effect of the dietary treatments on physical activity is shown in table 1. Diets were given once daily just after the darkness to light changeover. Rats receiving the restricted-calorie intake level consumed all of their diet during the first hour after feeding. Rats fed the casein diet at the high calorie level ate all of their diet during the first 8 hours after feeding. Rats fed the amino acid diet at the high calorie level ate all of the diet given them, but took 12 hours or more to do so. At either level of calorie intake there was no significant effect on physical activity due to substituting casein for amino acids as the nitrogen source, but there was a consistent tendency for the casein-fed rats to be more active, which bordered on significance. An analysis of variance, however, showed a highly significant effect ($P < 0.01$) of calorie intake on voluntary physical activity. This increase in activity due to caloric restriction appears to reflect an abolition of quiescence rather than an elevation of peak activity. During the 12 hours of darkness when the rat is normally most active (3), there was no difference in physical activity between groups. The 12 hours of light caused no appreciable change in the activity of the calorie-restricted animals, but the illumination caused a significant reduction in activity for rats fed at the high calorie level ($P < 0.01$). Thus, rats receiving the high calorie intake had about 70% of their activity at night, whereas calorie-restricted animals had nearly the same activity, day or night. Food deprivation appears to influence voluntary activity of the rat in a manner which overrides normal diurnal variation. The pattern of physical activity and the eating pattern appear to be related.

Carcass gains. The calorie, nitrogen, and ingesta-free weight gains are given in table 2. Calorie, nitrogen, and weight gains were significantly reduced ($P < 0.01$) by calorie restriction. Rats receiving casein at the higher calorie level gained more weight ($P < 0.05$) than amino acid-fed rats; although the change in nitrogen source had no statistically significant effect on calorie gain due to greater variability in the calorie gain data, there was a trend toward greater calorie gain in casein-fed rats, similar to the trend in our earlier report (1). An

TABLE 1

Diurnal variation in revolutions turned in activity cages over a 3-week period by rats fed nitrogen either as amino acids or as casein, at 2 levels of calorie intake

N source [1]	Intake [2]		Mean voluntary physical activity		
	N	Calories	12-hr light	12-hr dark	24-hr total
	g	*kcal*	*rev*	*rev*	*rev*
AA	8.59	1400	1379 ± 456 [3]	4143 ± 701	5523 ± 838
Casein	8.59	1425	1981 ± 465	4230 ± 736	6211 ± 997
AA	8.93	982	4584 ± 1023	3961 ± 417	8544 ± 1153
Casein	8.94	1040	6074 ± 1169	4964 ± 1050	11,038 ± 1158

[1] AA = amino acid mixture simulating casein.
[2] Mean dietary intake/rat over the entire 31-day experimental period.
[3] SE of mean; 6 rats/group.

TABLE 2

Weight gains, calorie and nitrogen storage of rats fed casein or a mixture of amino acids simulating casein at 2 calorie intake levels

N source [1]	Intake [2]		Weight gains [3]	Nitrogen stored	Calories stored
	N	Calories			
	g	*kcal*	*g*	*g*	*kcal*
AA	8.59	1400	78 ± 3.2 [4] *	3.35 ± 0.21	113.7 ± 11.4
Casein	8.59	1425	87 ± 1.6 *	3.59 ± 0.07	129.7 ± 9.4
AA	8.93	982	25 ± 3.5	1.59 ± 0.15	14.8 ± 5.5
Casein	8.94	1040	33 ± 2.3	1.87 ± 0.11	26.3 ± 3.3

[1] AA = amino acid mixture simulating casein.
[2] Mean dietary intake rat over the entire 31-day experimental period.
[3] Gain in ingesta-free carcass weight over the 31-day period.
[4] SE of mean; 6 rats/group.
* Adjoining means significantly different ($P < 0.05$).

analysis of variance showed a significant difference between casein and amino acid-fed rats in nitrogen gain per gram of digestible nitrogen intake ($P < 0.05$), with casein-fed rats being more efficient, and there was a trend toward greater nitrogen storage per gram of gross nitrogen intake in these rats. With large groups in the earlier study, differences in gross nitrogen storage were found to be significant.

Radiorespirometry studies. There was no significant effect of energy level or nitrogen source on the percentage of injected dose recovered in expired $^{14}CO_2$, feces, or urine during the 23 hours following injection of glucose-6-^{14}C and glucose-U-^{14}C (table 3). An analysis of variance shows that the nitrogen source in the diet had a significant effect ($P < 0.05$) on the percentage of injected glucose-1-^{14}C recovered as $^{14}CO_2$ (table 4). This is in agreement with our earlier report of differences in nitrogen storage at the high calorie intake level, since a change in the activity of the pathways of carbohydrate metabolism would be expected to alter protein metabolism as well. The significantly greater recovery of $^{14}CO_2$ from glucose-1-^{14}C in the amino acid-fed rats indicates greater use of alternative pathways of carbohydrate metabolism in these animals (8). The time the diet was fed since the last injection did not appear to affect the metabolism of the labeled glucose, as block effects were not significant. Despite reports of changes in the activity of the hexose monophosphate shunt due to calorie restriction (4, 5) our $^{14}CO_2$ recovery totals indicated no difference due to varying the caloric level (tables 3, 4). The explanation for this difference in results can best be explained by examining the pattern of $^{14}CO_2$ recovery plotted against time. Figure 1 shows the $^{14}CO_2$ recovery from glucose-1-^{14}C from the second to the twenty-fourth hour after feeding. An analysis of variance (table 4) of the percentage of injected glucose-1-^{14}C recovered as $^{14}CO_2$ after 6 hours shows a significant effect of calorie level ($P < 0.05$). This agrees with the earlier report of Lee and Lucia (4), who used a 3.5-hour collection period that calorie-restricted animals have a less active hexose monophosphate shunt. This measurement of activity of the hexose monophosphate shunt does not appear to reflect accurately the total amount of glucose metabolized by this route, at least for the casein-fed rats, since rats fed casein at the high calorie intake showed a reduced $^{14}CO_2$ release from the sixth to twenty-third hour after injection, whereas calorie-restricted animals fed casein maintained a steady rate of $^{14}CO_2$ production. As a result, the percentage of injected glucose-1-^{14}C metabolized to $^{14}CO_2$ after 23 hours was unaffected by calorie intake. The effect of changing nitrogen source in the diet on $^{14}CO_2$ production was significant at 6 hours after injection ($P < 0.01$) as well as after 23 hours ($P < 0.05$). An examination of the mean squares gives an indication of the greater magnitude of the effect of source of dietary nitrogen on glucose-1-^{14}C metabolism as compared with the effect of calorie restriction (table 4). An analysis of variance of $^{14}CO_2$ recovery after 6 hours from glucose-6-^{14}C and glucose-U-^{14}C showed no effect due to calorie level or nitrogen source, similar to the total 23-hour observations.

TABLE 3

Percentage of injected ^{14}C from radioactive glucose recovered in expired $^{14}CO_2$, feces, and urine during the first 23 hours following injection

N source [1]	Intake [2]		Glucose-1-^{14}C			Glucose-6-^{14}C			Glucose-U-^{14}C			
	N	Calories	$^{14}CO_2$	Feces	Urine	$^{14}CO_2$	Feces	Urine	$^{14}CO_2$	Feces	Urine	Urinary citrate [4]
	g	*kcal*	*% injected ^{14}C*			*% injected ^{14}C*			*% injected ^{14}C*			
AA	8.59	1400	67.7 ± 4.4 [3]	1.1 ± 0.3	1.9 ± 0.4	38.0 ± 4.0	2.9 ± 0.5	12.8 ± 4.1	53.7 ± 6.3	2.8 ± 0.7	14.6 ± 4.4	3.7 ± 1.2
Casein	8.59	1425	53.1 ± 4.8 [3] *	0.6 ± 0.2	1.9 ± 0.3	37.4 ± 7.4	3.4 ± 1.3	12.5 ± 2.7	52.8 ± 6.3	2.0 ± 0.4	14.3 ± 3.5	5.5 ± 2.4
AA	8.93	982	63.4 ± 3.9	0.9 ± 0.4	3.1 ± 0.4	46.7 ± 10.2	3.0 ± 1.1	16.6 ± 5.5	63.4 ± 8.4	2.1 ± 0.7	16.6 ± 4.9	2.6 ± 1.3
Casein	8.94	1040	56.6 ± 4.9	0.4 ± 0.1	2.0 ± 0.2	33.0 ± 3.5	2.6 ± 0.9	18.9 ± 3.9	46.2 ± 5.3	2.6 ± 0.7	19.3 ± 5.3	5.7 ± 2.1

[1] AA = amino acid mixture simulating casein.

[2] Mean dietary intake/rat over the entire 31-day experimental period.

[3] SE of mean; 6 rats/group.

[4] Specific activities (μCi/mEq) were: AA, 1400 kcal = 0.037 ± 0.012; casein, 1425 kcal = 0.057 ± 0.025; AA, 982 kcal = 0.024 ± 0.012; casein, 1040 kcal = 0.052 ± 0.019; none of these values proved to be significantly different from the others.

* Designated means significantly different ($P < 0.05$).

The differences in physical activity between day and night in rats receiving the high calorie intake might have been altered if the rats had been fed at night rather than the early morning, and this might have had an effect on metabolism. However, all rats in the present study were fed in the same manner and according to the same schedule so that the differences observed are valid within the confines of this particular schedule of care and feeding, which is that most commonly followed for laboratory rats.

We observed earlier that rats fed casein diets at a high calorie level consumed their diets more rapidly than rats fed a comparable amino acid diet (1). Tepperman and Tepperman (14) and Hollifield and Parson (15) reported evidence for greater activity of the hexose monophosphate shunt and greater fat deposition in rats trained to eat their food in a shorter period of time. This does not appear to be the reason for the greater use of the hexose monophosphate shunt in our amino acid-fed rats, therefore, since faster eating by the casein-fed rats would be expected to increase hexose monophosphate shunt activity in these animals. In addition, the $^{14}CO_2$ recovery data indicated a similar difference between casein- and amino acid-fed rats at a restricted level of intake (tables 3 and 4) where all rats consumed their diets in one hour or less.

These data are not consistent with the concept that voluntary activity reflects the state of energy metabolism in the animal. Although calorie restriction increased voluntary activity ($P < 0.01$), it had little effect on $^{14}CO_2$ recovery from injected glucose-1-^{14}C over a 23-hours period. Furthermore, glucose-1-^{14}C metabolism was affected most by substituting for casein in the diet a mixture of amino acids simulating casein. This change in nitrogen source had no significant effect on voluntary activity.

Urinary ^{14}C excretion. Table 3 lists the percentage of injected ^{14}C recovered in the urine during the 23 hours following administration of glucose-1-^{14}C, -6-^{14}C and -U-^{14}C. Although dietary treatment had no effect on urinary ^{14}C excretion, a greater percentage of the injected dose ($P < 0.01$) was excreted in urine when glucose-6-^{14}C or

TABLE 4

Mean squares of the analyses of variance for $^{14}CO_2$ recovery and urinary N

Source of variation	$^{14}CO_2$ recovered				Urinary N excretion
	Labeled carbohydrate administered				
	Glucose 1-^{14}C	Glucose 6-^{14}C	Glucose 1-^{14}C		
	(%/23 hr)[a]		(%/23 hr)[a]	(%/6hr)[a]	(mg/24 hr)[a]
Blocks	354.17	544.33	233.32	148.00	479
E = energy level	13.78	29.10	0.03	425.97*	16,970**
S = nitrogen source	485.55	308.24	688.33*	1,226.23**	1,883*
ES	397.97	256.04	89.12	10.28	170
Error	238.60	199.87	84.08	74.67	446

** Significant ($P < 0.01$).
* Significant ($P < 0.05$).

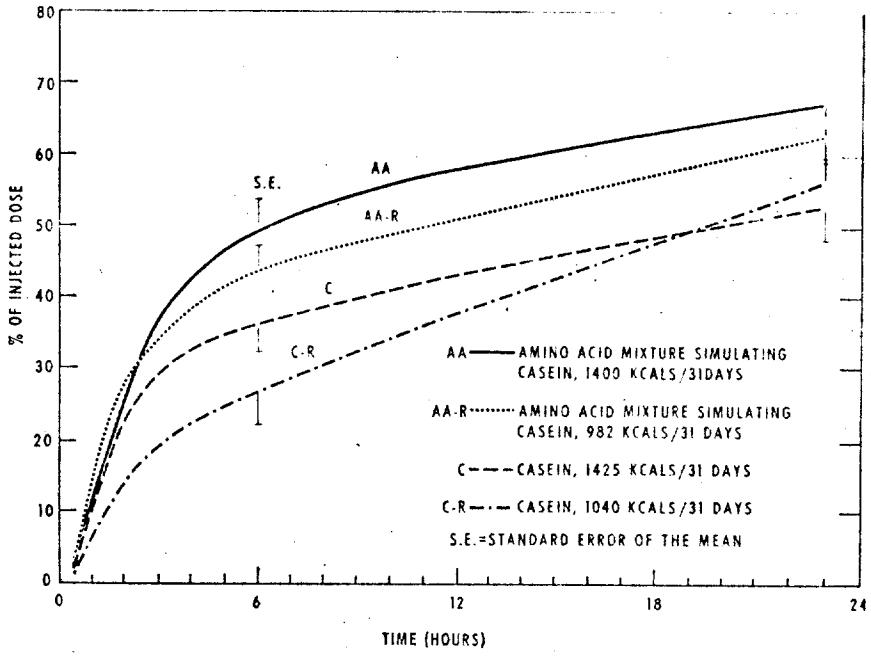

Fig. 1. Effect of calorie intake and nitrogen source on percentage of ^{14}C from intraperitoneally injected glucose-1-^{14}C recovered as $^{14}CO_2$.

glucose-U-^{14}C was given than when glucose-1 ^{14}C was the injected material. Urea ^{14}C accounted for only about 2% of urinary ^{14}C from glucose-6-^{14}C or glucose-U-^{14}C. Urease released about 20% of the urinary ^{14}C contributed by glucose-1-^{14}C, indicating that the bulk of the ^{14}C excreted was in other end products of metabolism.

Urinary ^{14}C excretion from glucose-6-^{14}C or glucose-U-^{14}C was similar, indicating that the terminal 5 carbons of glucose were metabolized by the same route. In fractionating the urinary ^{14}C activity, less than 5% was found in oxalic acid and between 5 and 10% was found in the ether extract, creatinine, osazone, and dicarboxylic amino acid fractions. The quantitatively greatest source of urinary ^{14}C was found to be citrate which accounted for about 30% of the urinary label and the percentage of injected glucose-U-^{14}C that was recovered as urinary citrate is reported in table 3.

Although no significant differences could be established due to the high variability in citrate-^{14}C excretion, a clear trend was evident indicating greater ^{14}C recovery in urinary citrate when casein was the nitrogen source rather than a mixture of amino acids simulating casein. The mean citrate excretion in the urine of rats consuming the 4 diets was nearly the same, ranging from 0.96 ± 0.11 mEq/day with the high calorie diet containing casein to 1.10 ± 0.12 mEq/day with low calorie diet containing casein. Bellin and Steenbock (16) earlier reported wide variation in citraturia regardless of dietary changes.

The specific activity of urinary citrate following glucose-U-^{14}C administration tended to be greater from rats fed casein than from those fed amino acids (table 3). If this trend is assumed to be real and urinary citrate is assumed to be representative of tricarboxylic acid cycle intermediates throughout the body, it appears that more of the ^{14}C from glucose is passing through the glycolytic scheme and into the tricarboxylic acid cycle in the casein-fed rats. This is consistent with the conclusion from the radiorespirometry data that rats fed amino acid diets derived a significantly larger portion of their energy from direct oxidation of glucose than casein-fed rats did. However, the possibility exists that the diammonium citrate in the amino acid diets diluted the ^{14}C activity in body citrate pools since the dietary intake of citrate was about 2 mEq/day and urinary output was about 1 mEq/day. The explanation for apparently greater use of the glycolytic scheme and tricarboxylic acid cycle in glucose metabolism of casein-fed rats is not clear. There are several possible explanations, including the following:

1) A dilution of the tricarboxylic acid cycle intermediates with intermediates from rapid amino acid breakdown might cause increased gluconeogenesis and a resulting inhibition of glycolysis causing glucose to be diverted to alternative pathways of metabolism. The normal route of glutamic acid breakdown involves the formation of α-ketoglutarate (17), whereas propionic acid formed in the degradation of methionine and threonine (18), serine, alanine, valine, and isoleucine (19) can be converted to succinate (20) in rather large quantities (21). Succinate and α-ketoglutarate thus formed could be metabolized via the tricarboxylic acid cycle. Gupta et al. (22), however, observed that free amino acids did not disappear from the digestive tract more rapidly than intact protein unless the protein was relatively insoluble (zein). This would indicate that amino acid absorption in rats fed casein or amino acids is not different.

2) The NH_4 citrate supplement added to amino acid diets to increase their N content might be metabolized by the tricarboxylic acid cycle and utilized for gluconeogenesis, resulting in inhibition of glycolysis and causing glucose to be diverted to alternative pathways of metabolism. A quantitatively great effect would not be expected, however, since the daily intake of NH_4 citrate by amino acid fed rats was about 0.16 g.

3) There are a number of recent reports in the literature that would suggest that the amino acid mixture used in this study, despite the fact that it simulates casein, does not contain adequate glutamic acid, glutamine, asparagine and arginine to support maximal weight gains of rats (23–26). Most workers who have fed "adequate levels" of dispensable and indispensable amino acids still obtain growth rates somewhat less than they obtain with protein-containing diets and thus the possibility of deficiencies of unknown factors such as strepogenin, postulated by Woolley (27), cannot be eliminated. Schwartz et al.[7] have indicated preliminary evidence for the existence of a previously unrecognized growth factor in 15% casein diets. The metabolic adaptation in direct oxidation of glucose observed in this study may be a consequence of a deficiency of either known or unknown substances.

The trend toward increased non-protein caloric storage (presumably fat) in young rats fed amino acid rather than casein diets reported in an earlier paper (1), might be due to the increased direct oxidation of glucose on amino acid diets and might be an adaptation to a deficiency.

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RAPID UPTAKE OF [1-^{14}C]ACETATE BY THE ADULT RAT BRAIN 15 SECONDS AFTER CAROTID INJECTION

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SUMMARY

[1-^{14}C]Acetate was injected into the carotid artery of adult rats followed by decapitation after 15 sec. Brain, liver and blood lipids were extracted. Analysis of the lipids showed the following:

1. There was considerably more uptake of radioactivity in the brain as compared to the liver or the plasma.

2. High radioactivity in the cholesterol fraction indicating rapid synthesis from radioactive acetate.

3. Incorporation of radioactivity into all major polar lipid fractions of the brain lipids including sphingomyelin and cerebrosides, considered to be myelin lipids. Phosphatidyl serine was the most highly labeled component.

4. Palmitic acid isolated from the brain was synthesized *de novo* from acetate and stearic acid was formed by chain elongation.

All these metabolic reactions occurring so rapidly in the brain are discussed in view of the older concept that adult brain is a tissue characterized by slow metabolism.

INTRODUCTION

Despite work throughout the last decade, in which numerous workers have shown appreciable amounts of incorporation of radioactive tracers into the brain, the general impression that the lipid metabolism of the brain is rather slow persists. Earlier literature[1] implied that ^{32}P injected intravenously or intraperitoneally was incorporated very slowly into brain lipids mainly because the blood brain barrier restricted the entry of the phosphate ion. However, once it permeated into the brain tissue there was rapid incorporation into phospholipids within 30 min. When heavy water[2], ^{2}H-labeled fatty acids[3,4], and cholesterol[5] were administered to adult rats, very

List of Abbreviations used: PC = Phosphatidyl choline; PE = Phosphatidyl ethanolamine; PS = Phosphatidyl serine; Cereb. = Cerebroside; Cera. = Ceramid; Cereb. Sulf. = Cerebroside sulfate;

little, if any, label was incorporated into brain total lipids, although very young rats took up the label rapidly. Discussing the biosynthesis and turnover rates, DAVISON[6] concluded that although the CNS contains large amounts of lipids, biosynthesis and mean turnover rates of typical myelin lipids including cholesterol are quite slow. Against this background a very recent communication from OLDENDORF[7] shows a rapid uptake of many essential amino acids only 15 sec after a carotid artery injection of the test compound. The present work was designed to examine in a similar way the rapid uptake and incorporation of [1-^{14}C] acetate, given *via* a carotid artery injection, by the adult rat brain.

MATERIALS AND METHODS

Animals

Six adult albino Wistar rats weighing approximately 400 g were used in the study; they had free access to food and water.

Tracer

10 μC of [1-^{14}C] acetate (spec. act. 45 mC/mM, New England Nuclear Corp., Des Plaines, Ill.) dissolved in 0.2 ml of water was injected into the carotid artery of the adult rats as one rapid bolus with decapitation 15 sec later as described by OLDENDORF[8]. Brain tissue was quickly dissected out, placed in a 3-ml syringe and extruded into a previously weighed vial containing 5 ml of chloroform–methanol (2:1, v/v). Similarly, a portion of the liver from each lobe was quickly added to another vial containing the same solvent. The total time between injection and extrusion of the tissue into chloroform–methanol was never more than 45–50 sec. Blood was collected into heparinized tubes and centrifuged immediately for 3 min. Plasma was separated and frozen in dry ice immediately until analyzed.

Extraction of the lipids from brain, liver and plasma was done by the method of FOLCH *et al.*[9]. The total lipids were extracted from the tissue of the six rats separately and mean values with standard deviations are given in Table I. The fractionation of the lipids was done on pooled samples. Fractionation of total lipids, isolation of pure palmitic and stearic acid, decarboxylation of the pure fatty acids[10] *etc.* has been described in earlier communications[11,12]. A packard TriCarb liquid scintillation spectrometer Model 574, operating at 80% efficiency was used for all radioactivity determinations.

RESULTS

TABLE I

INCORPORATION OF [1-^{14}C] ACETATE BY RAT BRAIN, LIVER AND PLASMA LIPIDS 15 SEC AFTER CAROTID ARTERIAL INJECTION

	Spec. act. total lipids (counts/min per mg)	*Radioactivity of total lipids (counts/min per g fresh weight of tissue)*	*% of given dose (μC/100 g of body weight) recovered in total lipids*	*Cholesterol spec. act. (counts/min per mg)*	*Polar lipids (phospholipids + sphingolipids) (counts/min per mg)*
Blood plasma	168 ± 121	407	0.008	—	—
Brain	320 ± 01	34828	0.64	80	294
Liver	31 ± 25	1241	0.02	105	54

Table I shows the data regarding uptake and incorporation of radioactivity from injected [1-^{14}C]acetate. The values represent the average of six determinations worked out separately. The specific activity of total lipids of the brain was much higher than those of the liver or the plasma. The total radioactivity expressed as counts/min of total lipids per g of wet brain is about 30 times higher in the brain than in the liver. The neutral lipid fraction from the brain was less than 1% of the brain total lipids but had extremely high radioactivity. This fraction was not characterized in the present study. The polar lipids of the brain, comprising phospholipids and sphingolipids, had higher radioactivity than cholesterol, whereas the opposite was true in the case of the liver.

Table II shows the radioactivity values of various polar lipid fractions isolated by column chromatographic methods. The data obtained 24 h after intraperitoneal injection of acetate are included for comparison. It may be seen that the interval between administration of dose and sacrifice of the animal has a profound influence on the pattern of labeling in so far as the specific activities of various components are concerned. The specific activity of phosphatidyl choline was highest in the 24-h experiment whereas phosphatidyl serine had maximum specific activity in the 15-sec experiment. It may be noted here that both sphingolipids, cerebrosise and sphingomyelin, had higher specific activity after 15 sec than after 24 h despite the fact that an approximately 100-fold higher dose of radioactive acetate was injected intraperitoneally in the 24-h experiment.

TABLE II

SPECIFIC RADIOACTIVITY OF POLAR LIPIDS AFTER ADMINISTRATION OF [1-^{14}C]ACETATE

	Specific activity (counts/min per mg)		
	Brain		*Liver*
	24 h	*15 sec*	*15 sec*
Phosphatidyl choline	130	80	43
Phosphatidyl ethanolamine	75	45	35
Phosphatidyl serine	46	239	5
Sphingomyelin	30	59	15
Ceramid	—	138	—
Cerebroside	51	138	—
Cerebroside sulfate	—	9	—

The specific activities of the various phospholipids obtained by fractionation of liver total lipids shows that phosphatidyl choline was the most active fraction and the pattern is somewhat similar to that of the brain seen in the 24-h experiment but very much different from that of the brain in the 15-sec experiment.

The percent distributions of radioactivity of palmitic and stearic acids isolated from the brain total fatty acids are shown in Table III. The distribution of radioactivity in palmitic acid is almost identical irrespective of the period between injection of the radioactive tracer and sacrifice of the animal. If palmitate is synthesized *de novo* from [1-^{14}C]acetate every odd carbon is labeled and the carboxyl carbon would have 1/8 or 12.5% of the total activity[12]. The % relative carboxyl activity of brain palmitic acid is very close to the calculated value of *de novo* synthesis of palmitate. Higher activity in the carboxyl carbon of stearic acid indicates chain elongation and there is a steady decrease in the % relative carboxyl activity as the interval between administration of the dose and sacrifice of the animal increases.

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN PALMITIC AND STEARIC ACID AFTER INJECTING [1-^{14}C]-ACETATE i.p., intraperitoneal; i.c., intracarotid; RCA, relative carboxyl activity (radioactivity in –COOH·100/radioactivity in total fatty acids).

Dose given	*Period between injection of dose and sacrifice of rats*	*Fatty acid isolated from brain total lipids*			
		Palmitic acid		*Stearic acid*	
		Spec. act. (counts/min per mg)	*% RCA*	*Spec. act. (counts/min per mg)*	*% RCA*
[1-^{14}C]-Acetate (i.p.)	24 h	—	14.1	—	20.6
[1-^{14}C]-Acetate (i.p.)	4	—	13.5	—	25.0
[1-^{14}C]-Acetate (i.c.)	15 sec	595	13.4	91	43.1

DISCUSSION

The earlier findings regarding relatively very slow uptake of intravenously administered ^{32}P (see ref. 1) were explained not so much as due to slow metabolism of the brain but to the slow penetration of ^{32}P across the blood brain barrier[13]. KISHIMOTO AND RADIN[13] came to the conclusion that in adult animals, the blood brain barrier causes the brain to come out second best to liver when there is a competition for intraperitoneally administered isotopic precursors. For this reason most workers prefer to use young rats, 10–15 days old. Thus, in a classical experiment to show the inertness of brain myelin cholesterol DAVISON AND WAJDA[13] injected 4-^{14}C-cholesterol into rabbits and chickens soon after birth and found the label in the same position even after as long a period as 375 and 502 days, respectively. Thus, while observing slow uptake or persistance of the label, the age of the experimental animal[11], as well as effect of the blood brain barrier on the penetration of the compound has to be considered[28]. In a series of papers from this laboratory[11,16,17] we have deliberately chosen adult animals and shown a direct uptake of long chain fatty acids, both saturated and polyunsaturated. The uptake of radioactivity in all these experiments was considerably higher in the liver lipids than in the brain, which agrees with the conclusions of KISHIMOTO AND RADIN[13]. However, we now feel that the time interval and route of administration are also very important factors. LEVIN AND KLEEMAN[18], emphasizing the routes of entry of compound into the brain, have stressed the importance of the sink action of CSF when compounds are presented to the brain by way of the blood.

The route of entry chosen in the present work does seem to have a profound effect on the uptake of radioactivity. In the present study, the tracer was injected directly into the common carotid artery in one quick pulse so that the brain would have the first chance at the metabolic turnover of the tracer. During the next 15 sec before decapitation, the blood flow was unimpeded. This would ensure flushing of any tracer still remaining in the blood capillary and not taken up by the brain. OLDENDORF[8] has calcualted that approximately 8% of the injection is distributed to the brain. Thus, out of the 10 μC of 1-^{14}C acetate that was injected, only a little over 1 μC actually was available to the brain. In spite of this small amount, the brain total lipids incorporated a fairly good amount of the label and, of more significance, the

specific activity of brain lipids was considerably higher than that of the liver total lipids. In the adult animals this is the first time that we have observed this relationship. The total ^{14}C radioactivity in the brain per g of tissue is about 28 times higher than that in the liver. Part of this could be due to differences in the blood flow (ml/min per g) to the two organs. The total radioactivity of blood lipids per ml of plasma was so low that it could not account for the total radioactivity of brain lipids per g of tissue, which rules out contamination from trapped blood.

Another significant fact was the extremely short interval of 15 sec between administration of the tracer and decapitation and 45 sec before the tissue was extruded into chloroform–methanol, thus preventing any further metabolic reactions in the dissected tissue. It was observed that within this very small interval, radioactive acetate was incorporated into brain total lipids, including cholesterol. The rapid synthesis of cholesterol, as evidenced by fairly high specific activity in the adult animal, confirms the observations of KABARA[19], who showed that there must be several compartments of sterol metabolism, one or more of which has a very rapid turnover.

The radioactivity from 1-^{14}C acetate was incorporated into all major polar lipids of the brain within 15–45 sec. Cerebroside, sphingomyelin, and cholesterol have been classified as typical myelin lipids[20,21]. Activity from 1-^{14}C acetate was found in these fractions also within the short interval. In a recent paper AGRAWAL *et al.*[22] have reported incorporation of 1-^{14}C acetate into myelin lipids within 10 min, but the animals used were only 16 days old.

The differences in the specific activities of various polar components of brain lipids in the 24-h experiment as compared to the 15-sec experiment shows that the turnover rates of these fractions must be different. This observation does not agree with that of DAVISON[6], who has proposed that all myelin sheath constituents (proteins and lipids) have the same turnover rate. However, it may be pointed out that extremely short periods between injection and sacrifice of animals are considered in the present study unlike the long periods considered in the study by DAVISON[6]. SMITH[23] has found that for periods ranging from 24 h to 56 days different myelin constituents turn over at distinctly different rates. For example she found that inositol phosphatide showed the highest degree of metabolic activity, where as sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine and cerebroside incorporated radioactive carbon to a moderate extent. This agrees with the *in vitro* work by YAGIHARA *et al.*[24].

In the present study, phosphatidyl serine was the most radioactive component in the brain polar lipids, when the animals were decapitated within 15 sec, but not if the animals were killed after 24 h. ABDEL-LATIF AND ABOOD[25] in their studies on developing rat brain found that radioactivity from U-^{14}C serine was incorporated mostly in phosphatidyl serine. If the serine in phosphatidyl serine had been decarboxylated, it would have given rise to high activity in phosphatidyl ethanolamine. That such reactions are not major pathways in the brain was further confirmed from the work of ANSELL AND SPANNER[26]. This may explain the low activity of phosphatidyl ethanolamine in the 15-sec experiment as against high radioactivity of phosphatidyl serine fraction; but the high radioactivity observed in phosphatidyl serine fraction itself needs further work for elucidation.

More recently, ANSELL AND SPANNER[27] have referred to a significant turnover rate of the ethanolamine moiety in brain myelin.

The theoretical value of % relative carboxyl activity for the *de novo* synthesis of palmitic acid is 12.5% which is very close to the experimental value obtained in the 15-sec experiment. Thus, one can conclude that within this short period palmitic acid was synthesized *de novo* from [1-^{14}C]acetate. Chain elongation of the palmitic acid to stearic acid by addition of an acetate unit is also seen within 15 sec, since the experimental value of 43.1% relative carboxyl activity is much higher than the theoretical value of 11.1% for the *de novo* synthesis of stearic acid.

In conclusion, the present study shows a substantial uptake of [1-^{14}C]acetate injected intracarotid by the adult rat brain within a very short period of 15 sec between injection and decapitation. Further, the radioactive carbon was incorporated into major polar lipid fractions including cholesterol, cerebroside and sphingomyelin considered as typical myelin lipids.

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Hypothalamic Obesity: The Myth of the Ventromedial Nucleus

Abstract. *Lesions restricted to the ventromedial nucleus of the hypothalamus were neither necessary nor sufficient for, and did not contribute to, the production of hypothalamic obesity. Hypothalamic lesions and knife cuts that do produce obesity damage the nearby ventral noradrenergic bundle or its terminals.*

For over 30 years the ventromedial nucleus of the hypothalamus (VMN) has been linked in theory to the suppression of eating. There have been many reports of hyperphagia and obesity after destruction of the VMN (*1*). Both neurophysiological and anatomical evidence for connections between a presumed VMN satiety center and a lateral hypothalamic feeding center have been reported (*2*).

However, there is evidence that the overeating and obesity that once seemed associated with destruction of the VMN is not due to VMN damage per se, but rather to destruction of the nearby ventral noradrenergic bundle (*3*). The ventral noradrenergic bundle ascends from brainstem nuclei to innervate limbic areas, including several hypothalamic loci, but sends relatively few terminals to the VMN (*4*).

That VMN damage itself contributes to hypothalamic obesity is open to question. Lesions of the VMN that are produced by radio-frequency currents fail to produce obesity (*5*). Closer examination of the studies that do link VMN lesions to obesity reveals that the effective lesions overflow the bounds of the VMN, the largest lesions typically producing the fattest rats (*1*). Finally, lesions caudal or lateral to the VMN, parasagittal knife cuts rostrolateral to the VMN, and midbrain lesions can all produce obesity even though the VMN is left intact (*1*, *6*).

I now report that even under optimal testing conditions lesions restricted to the VMN, even iron depositing lesions (*5*), produce neither overeating nor obesity. The VMN lesions cause obesity only when they overflow the VMN, and the magnitude of the obesity is proportional to the amount of overflow.

Female albino rats ($N = 119$) were allowed free access to a highly palatable high fat diet (*7*) and tap water. Lesions were produced by passing an anodal direct current through platinum-iridium, stainless steel, or iron wire electrodes. The lesions were all aimed at the rostral tip of the VMN, with the use of stereotaxic coordinates that had previously been associated with rapid weight gains (*8*).

For the initial series of rats the bilateral lesions were produced by a current of 2 ma for 20 seconds (40 millicoulombs) as described in (*7*, *9*). The lesions that resulted from 40 mcoulomb were enormous (Fig. 1L), with iron electrode lesions by far the largest, and platinum-iridium the smallest (*10*). The lesioning dosages for subsequent groups of rats were therefore halved to 20 mcoulomb and for the iron and steel electrodes, halved again to 10 mcoulomb. Finally, to approximately match the size of the very large lesions produced by 40 mcoulomb delivered by iron or steel electrodes, platinum electrode lesions were made with 160 mcoulomb (*11*).

Lesions that fell entirely within the borders of the VMN and the intervening midline area (Fig. 1F) did not produce obesity. Weight gains for the five animals with these lesions fell within the range (0 to 1.6 g/day) of 17 sham-operated controls. Lesions that damaged both the VMN and the dorsomedial nucleus also did not cause obesity (Fig. 1C). In contrast, lesions that extended ventrally from the VMN did produce slight obesity (3.0 g/day) (Fig. 1G). This slight effect appears to be due to the damage ventral to the VMN. The failure to produce obesity with lesions completely restricted to the VMN occurred despite the use of all of the parameters that maximize postlesion weight gains, that is, female rats (*7*), heavy iron deposits from anodal current delivered through iron or steel electrodes (*5*), and a palatable high fat diet (*7*).

The brain areas destroyed by the 55 smallest lesions were compared. There was a common area for the lesions of the five rats with the greatest weight gains (9.0 to 12.6 g/day). These most effective of the smallest lesions all destroyed an area immediately rostral to the rostral tip of the VMN (Fig. 1A). It is precisely across this area that a group of noradrenergic fibers crosses the midline within the suprachiasmatic decussation. These noradrenergic fibers are thought to derive from the ventral ascending noradrenergic bundle (*4*). Small lesions located more dorsally or more caudally were less effective (Fig. 1, B and D) (*12*).

Larger lesions produced far greater weight gains. If the thalamus and the nigro-striatal dopamine pathway at the extreme lateral edge of the hypothalamus (*4*) were spared, then the bigger the lesion, the greater the initial rate of weight gain. For example, a large platinum electrode lesion spared the VMN but produced rapid weight gains of 10.6 g/day (Fig. 1E). The correlation between lesion size and weight gain is illustrated by the representative series of lesion reconstructions in Fig. 1, F to L.

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8. With the top of the incisor bar 3.0 mm below the ear bar center, the electrode tip was placed 6.5 mm anterior to the ear bar center, 0.5 mm lateral, and 8.1 or 8.3 mm below the dura. The conical exposed electrode tips were 0.5 mm long.
9. To determine the influence, if any, of lesioning current (as opposed to the total amount of millicoulombs delivered), half of these rats received the same 40 mcoulomb slowly as 100 μa for 400 seconds.
10. In order to produce stainless steel and platinum-iridium electrode anodal lesions of equivalent size, the number of coulombs is tripled when going from stainless steel to platinum-iridium.
11. The size of the lesions was determined without reference to weight gains, by projecting appropriately magnified cresyl violet–stained sections directly onto the frontal plates of the Konig and Klippel atlas and then tracing at 0.4-mm intervals the absent normal tissue. I used all of the even-numbered atlas plates through the extent of the lesion. Tracing the absent normal tissue instead of the periphery of the apparent lesion scar corrected for shrinkage into the lesion cavity. Shrinkage and scar tissue were greatest with the iron and steel lesions, especially the iron.
12. The superachiasmatic nucleus is at least partly included within the area damaged by all of the five most effective lesions, and it relays fibers from the eye that regulate circadian cycles [R. Y. Moore, *J. Comp. Neurol.* 140, 1 (1972)]. Disruption of circadian cycles may in turn mediate hypothalamic obesity (R. M. Gold and G. Kapatos, manuscript in preparation).
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20. Chemically blocking the nocturnal alpha-adrenergic transmission with phentolamine produces eating, perhaps by the same mechanisms whereby electrolytic lesion blockade produced overeating and obesity [D. L. Margules, *J. Comp. Physiol. Psychol.* 73, 1 (1970)]. Conversely, beta-adrenergic stimulation during the day can be satiating [S. F. Leibowitz, *Nature* 226, 963 (1970)].

21. P. M. Quackenbush provided technical assistance, and G. Kapatos reviewed the manuscript. A preliminary report of this work was presented to the Eastern Psychological Association, Washington, D.C., May 1973.

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The wide area encompassed by the most effective lesions includes the wide area in the rostral hypothalamus to or through which the ventral ascending noradrenergic bundle projects (*4*). Many of the most effective lesions extended so far laterally that they appear to have damaged the medial edge of the ventral bundle itself (Fig. 1, K and L), thus producing especially rapid weight gains as high as 18.8 g/day. When the lesion was even larger than this (Fig. 1M) the nigro-striatal bundle (*4*) was damaged, and weight gains were minimal. This would be expected, since nigro-striatal lesions produce aphagia (*13*).

I found that when obesity is produced with parasagittal knife cuts (*14, 15*), the greatest weight gains require long cuts. Thus 3-mm cuts are more effective than 2-mm cuts which are more effective than 1-mm cuts (unpublished data). It thus appears that fibers projecting diffusely to more than one area are involved in hypothalamic obesity. The parasagittal knife cuts are only effective if they include the area lateral to the dorsal supraoptic commissure (Ganser), precisely the frontal level of the most effective small lesions of Fig. 1 (*15*). The parasagittal knife cuts that produce obesity apparently sever noradrenergic fibers as they turn medially from the ventral bundle to innervate numerous rostral hypothalamic structures. In further support of the notion that ventral bundle damage mediates hypothalamic obesity, it has been shown that obesity results from combining a unilateral parasagittal knife cut (or unilateral medial hypothalamic lesion) with a contralateral ventral bundle lesion (*3*).

It is perplexing that electrolytic or chemical damage to the ventral noradrenergic bundle or to its terminal areas produces overeating, while electrical or noradrenergic stimulation of the same loci also produces eating. Booth (*16*) has observed that the loci (presumably terminals) at which noradrenergic stimulation produces eating lie rostral to the loci (presumably ascending fibers) at which electrical stimulation produces eating. Other reports have localized the optimal locus for eating induced by electrical stimulation at the paraventricular nucleus (*17*). This structure receives a major proportion of the hypothalamic noradrenergic terminals (*4, 16*). Even the release of endogenous transmitter by small localized 6-hydroxydopamine infusions into the hypothalamus can produce eating (*18*).

A resolution of the paradox whereby lesioning or stimulation of the ventral bundle both produce eating may lie in the recent demonstration by Margules *et al.* (*19*) that norepinephrine applied to the perifornical medial forebrain bundle (ventral noradrenergic bundle) can either enhance or suppress eating depending on when it is administered during the daily circadian cycle. During the day norepinephrine does enhance eating, but at night it suppresses eating (*20*).

In conclusion, many of the lesioning or stimulating procedures that produce excessive eating appear to share in common damage, blockade, or stimulation to the ventral ascending noradrenergic bundle or its terminals. The VMN is merely a prominent landmark in the vicinity of effective loci.

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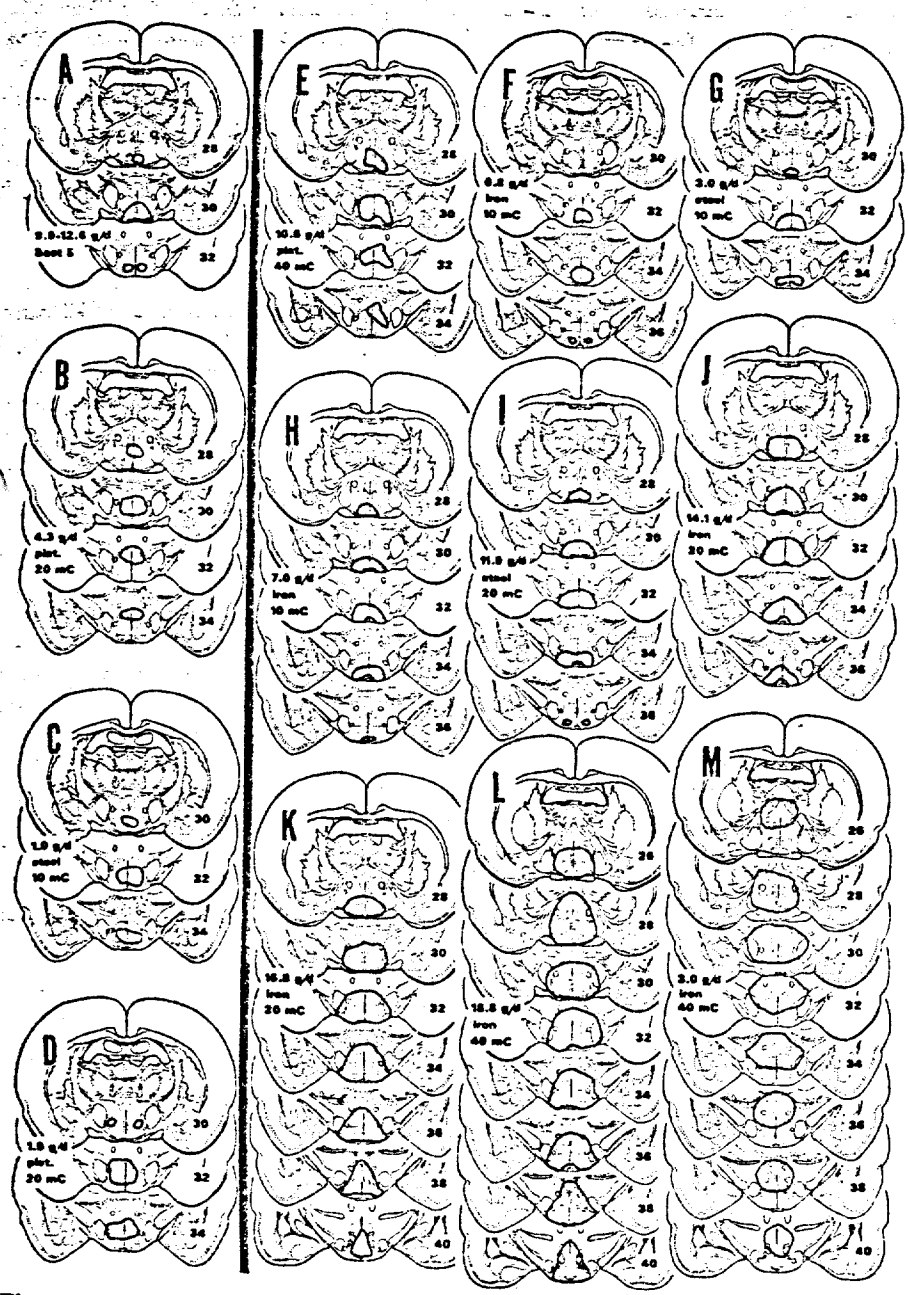

Fig. 1. Reconstructions of representative lesions, prepared as described in (*11*), and superimposed on even-numbered plates (plates 26 to 40) modified from the Konig and Klippel rat brain atlas. Weight gains per day for 2 weeks after surgery were computed as in (*7*). The VMN appears in plates 32 through 40. (A) Common area of destruction for the five greatest weight gains among the 55 smallest lesions. At level 30, the largest of these five lesions only slightly exceeded the common area. For (B) to (M) see text. Abbreviations: *g/d*, grams per day; *mC*, millicoulombs.

Comparative Effects of Casein and Amino Acid Mixture Simulating Casein on Growth and Food Intake in Rats

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ABSTRACT In several feeding methods, the effect of the amino acid mixture simulating casein on rat growth was compared with that of intact casein. When diets were provided ad libitum, weight gain and food intake were lower for rats fed amino acid diets than those fed a casein diet at dietary nitrogen levels of 3.2 and 4.8%, whereas no difference was observed between both the nitrogen sources at 0.8 and 1.6% levels. The superiority of casein was also found in pair-feeding experiments in which casein diet was pair-fed with amino acid diet on a daily basis at a nitrogen level of 3.2%. When animals were space-pair-fed for 1 hour twice daily, however, both diets supported identical growth during the 14-day experimental period. Identical growth rates were also obtained by force-feeding the same amounts of both diets. These results indicate that the amino acid mixture simulating casein is nutritionally equivalent with intact casein under conditions of space-feeding twice daily to pair-fed partners. J. Nutr. *103:* 1709–1715, 1973.

INDEXING KEY WORDS amino acid mixture · casein · weight gain · food intake · space-pair-feeding

Since the discovery of threonine in 1935, it has been demonstrated that human beings, as well as experimental animals, can grow and be maintained in positive nitrogen balance on amino acid diets. By employing amino acid mixtures it became possible to prepare the experimental diet in any desirable amino acid pattern, permitting thereby a more searching inquiry into the nutritional role of each amino acid. With the aid of the knowledge accumulated in earlier investigations, Greenstein, Winitz, and their colleagues (1, 2) developed a nutritionally complete amino acid diet which could maintain normal human subjects in a satisfactory nutritional state without untoward physiological and psychological response in long-term studies. Moreover, the recent improvement of production and supply conditions of various amino acids permits the new practical use of purified amino acid diets for treatment in some undernourished patients afflicted with impaired digestion and absorption. The chemically defined amino acid diets have also been shown to be very useful for the nutritional therapy of inflammatory bowel disease due to the fact that the diets are completely absorbed in the upper sections of intestine. The reduction of bulk and intestinal gases provides ideal conditions for endoscopic examination of the gastrointestinal tract. Moreover, the synthetic diets can offer nutritional support in pre- and postabdominal surgery (3–5).

When dietary protein is replaced with an amino acid mixture, one of the most important problems is the comparative nutritional efficacy of a protein and that of the corresponding amino acid mixture. Although many investigations on nutritional comparison of an amino acid mixture with intact protein have been reported, the studies using an amino acid mixture simulating intact protein are few. Among the human studies, Anderson et al. (6) ob-

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served no significant difference in nitrogen balance during the periods in which intact casein and the simulating amino acid mixture were given. On the other hand, when egg protein was replaced with the equivalent amount of amino acids, less positive nitrogen balance was observed in young male adults (7). Animal experiments showed that the amino acid mixture simulating casein was utilized as efficiently as intact casein by weanling rats (8) and adult rats (9). Nakagawa and Masana (10) also observed in experiments using littermate female rats that the amino acid mixture simulating casein supported growth and life span close to those obtained with intact casein. Likewise, in mouse growth studies, nutritional equivalence of crystalline β-lactoglobulin and the corresponding amino acids was reported (11). On the other hand, some investigators observed the superiority of intact protein in rat or hen experiments (12–14). Ahrens et al. (14) compared the nitrogen retention of rats fed intact casein with that of rats fed the amino acid mixture simulating casein at different levels of nitrogen and energy intake. From the results they concluded that casein was superior for the nitrogen retention at higher energy intake in young rats and at higher nitrogen intake in adult rats. Thus, in previous comparative investigations, agreement was not obtained because of differences in experimental conditions and animal species employed by the respective investigators. To elucidate the nutritional differences of protein and an amino acid mixture, we have carried out studies on comparison of the effects of casein and the corresponding amino acid mixture on rat growth and food intake in several feeding methods.

The final purpose of our studies on amino acid nutrition is to develop a nutritionally complete amino acid diet which supports the maximum growth rate and nitrogen retention obtained by diets containing high quality proteins.

METHODS

Diets and animals. The compositions of casein diet (N, 3.2%) and the corresponding amino acid diets (each containing 3.2% nitrogen) are shown in tables 1 and 2. The other diets of various nitrogen levels were prepared by varying the amounts of casein or amino acid mixture at the expense of sucrose. The amino acid pattern in the mixture (table 2) was based on the analysis of casein. Amino acids in the acid hydrolysate of casein were determined on an amino acid analyzer [1] except methionine, cystine, and tryptophan. The S-amino acids were analyzed after oxidation with performic acid as described by Moore (15) and tryptophan was analyzed colorimetrically with *p*-dimethylaminobenzaldehyde (16). The composition of amino acid diet B was the same as that of diet A except for partial substitution of aspartic acid with asparagine which was reported to be necessary for the maximal growth of rats (17, 18). Diammonium citrate was added to make the amino acid diets isonitrogenous with the casein diet.

Male rats of the Wistar strain weighing from 50 to 80 g were used in all experiments other than force-feeding experiments in which rats weighing from 100 to 130 g were used. They were separated into groups of eight rats each and housed in individual wire cages in a constant-temperature room (23 ± 1°) lighted from 8:30 AM to 8:30 PM. Individual weight gain and food intake were recorded daily.

Feeding methods. The experimental design included the following five different feeding methods.

Ad libitum feeding: Rats were fed diets ad libitum.

Pair-feeding: Rats were separated into two groups. Diets were pair-fed to each partner on a daily basis, i.e., each rat in one group was fed amino acid diet ad libitum, and its partner in the other group was fed the same amount of casein diet. In these experiments, diets kneaded into dumplings with an equal weight of distilled water were fed in clean porcelain cups. Food intake was calculated on an air-dried basis.

Space-feeding: Three space-feeding methods were used. In one method, diets were supplied for 2 hours once daily (at 9:00 AM). In the other methods, diets were given for 1 hour twice (at 9:00 AM and 5:00 PM) or thrice (at 8:30 AM, 2:00 PM, and 7:30 PM) daily. Prior to the regular feeding period, the rats were trained to

[1] Hitachi Amino Acid Analyzer KLA-3B, Hitachi Ltd., Tokyo, Japan.

TABLE 1

Composition of casein and amino acid diets of 3.2% nitrogen content

	Diet A	Diet B	Diet C
	%	%	%
Casein[1]	—	—	23.3
Amino acid mix[2]	25.8	25.1	—
Sucrose[3]	19.7	20.4	21.7
Cornstarch[4]	40.0	40.0	40.0
Soybean oil	8.0	8.0	8.0
Salt mix[5]	4.0	4.0	4.0
Cellulose[6]	2.0	2.0	2.0
Vitamin mix[7]	1.0	1.0	1.0

[1] Casein was purchased from Long Warry & District, Dairymen's Co. Assn. Ltd., Australia. The commercially available casein was confirmed to give the same growth rate as that obtained with the purified casein in rat growth experiments. [2] The composition of amino acid mixture is shown in table 2. [3] Sucrose was added as required to complete the mixture to 100%. [4] Cornstarch was replaced by α-cornstarch in pair- and space-pair-feeding experiments. [5] Salt mixture provided the following amounts of the salts/100 g of diet: (in g) NaCl, 1.002; KH_2PO_4, 1.372; $CaCO_3$, 1.171; $MgSO_4 \cdot 7H_2O$, 0.399; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.025; $CuSO_4 \cdot 5H_2O$, 0.006; $MnSO_4 \cdot H_2O$, 0.005; and (in μg) $ZnCl_2$, 800; KI, 20; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 100. [6] Cellulose powder of 100–200 mesh was purchased from Toyo Roshi Co., Ltd., Tokyo, Japan. [7] Vitamin mixture provided the following amounts of the vitamins/100 g of diet: retinyl acetate, 2000 IU; ergocalciferol, 200 IU; and (in mg) all-*rac*.-α-tocopherol, 10; thiamin·HCl, 0.59; riboflavin, 0.59; nicotinic acid 2.94; Ca pantothenate, 2.35; pyridoxine·HCl, 0.29; menaquinone, 0.06; myo-inositol, 11.76; ascorbic acid, 5.88; and (in μg) biotin, 10; folic acid, 20; cyanocobalamin, 20.

TABLE 2

Composition of amino acid mixture in diets A and B

Amino acid	Diet A	Diet B
	%	%
L-Arginine·HCl	0.95	0.95
L-Histidine·HCl·H_2O	0.93	0.93
L-Isoleucine	1.03	1.03
L-Leucine	1.87	1.87
L-Lysine·HCl	2.00	2.00
L-Methionine	0.63	0.63
L-Cystine	0.08	0.08
L-Phenylalanine	1.04	1.04
L-Tyrosine	1.10	1.10
L-Threonine	0.87	0.87
L-Tryptophan	0.22	0.22
L-Valine	1.28	1.28
L-Alanine	0.81	0.81
L-Aspartic acid	1.41	0.56
L-Asparagine	—	0.85
L-Glutamic acid	4.44	4.44
Glycine	0.37	0.37
L-Proline	2.11	2.11
L-Serine	1.11	1.11
Diammonium citrate	3.54	2.81
Total	25.79	25.06

consume the diet in a short period at the same intervals as those in the respective space-feeding method.

Space-pair-feeding: The same method as that used for pair-feeding was applied to space-feeding twice a day. Therefore, each rat of the casein group had identical intake with that of the corresponding animal of the amino acid group in all meals.

Force-feeding: Diets were suspended in distilled water (0.5 g of diet/ml) and force-fed via stomach tube thrice daily (at 8:30 AM, 2:00 PM, and 7:30 PM). Daily food intake was 9.5 g for each animal of both casein and amino acid groups.

Tap water was supplied ad libitum in all the experiments.

Statistical analysis. Data were treated statistically using Student's *t* test (19).

RESULTS

Ad libitum feeding experiments. Daily weight gain and food intake of growing rats fed amino acid diet A were compared with those of rats fed casein diet C at various dietary nitrogen levels. The results are shown in figure 1. At dietary nitrogen levels of 0.8 and 1.6%, there was no significant difference ($P > 0.05$) in weight gain and food intake whether diet A or diet C was given. However, diet C (3.2% nitrogen content) provided a significantly greater weight gain ($P < 0.01$) over iso-

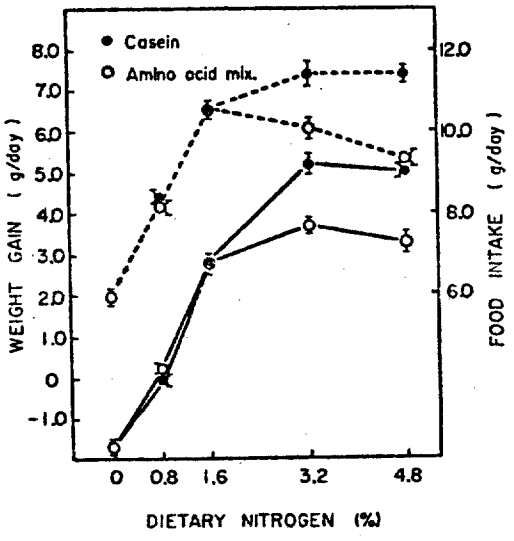

Fig. 1 Average weight gain (——) and food intake (- - - -) of rats fed casein diet C or purified amino acid diet A for 14 days. Each point represents the mean ± SEM of eight animals.

nitrogenous diet A, although increasing the nitrogen content of diets from 1.6 to 3.2% improved the growth rate of rats in both the dietary groups. An increase in food intake of rats fed diet C was observed at a dietary nitrogen level of 3.2% as compared to the 1.6% level, but food intake of rats fed diet A decreased slightly with the increase in dietary nitrogen level. The differences in growth rate and food intake between rats fed diet A and diet C were also significant ($P < 0.01$) at a dietary nitrogen level of 4.8%.

The amino acid composition of diet A was mainly based on the analysis of the acid hydrolysate of casein. Asparagine and glutamine, components of casein, were replaced with aspartic acid and glutamic acid, respectively, in diet A. Effects of the partial substitutions of asparagine for aspartic acid and glutamine for glutamic acid were tested separately and in combination with each other because dietary asparagine and glutamine were reported to play special roles for growth of rats fed amino acid diet (17, 18, 20). As shown in table 3, the substitution of asparagine for aspartic acid led to a slight improvement in growth with an increase of food intake. Although the difference was not significant ($P > 0.05$), the effect of dietary asparagine was always observed in repeated experiments. However, weight gain of rats fed amino acid diet supplemented with asparagine was still considerably less than that of rats fed casein diet. On the other hand, the substitution of glutamine for glutamic acid did not stimulate growth. From the results, the amino acid mixture of diet B containing asparagine was adopted as a mixture simulating casein, and its nutritional effect was compared with that of intact casein at a dietary nitrogen level of 3.2% in the following experiments.

TABLE 3

Effect of substituting asparagine for aspartic acid and glutamine for glutamic acid on rat growth

Diet	Weight gain	Food intake
	g/day	*g/day*
Casein diet (diet C)	5.2±0.2[1]	11.2
Amino acid diet (diet A)	3.4±0.3	9.2
Amino acid diet, asparagine[2] (diet B)	3.7±0.1[3]	9.9
Amino acid diet, glutamine[4]	3.4±0.3[3]	9.0
Amino acid diet, asparagine[2] and glutamine[4]	3.7±0.2[3]	9.8

[1] Mean ± SEM of eight animals. [2] Aspartic acid (60%) in diet A was replaced by asparagine. [3] Not significantly ($P > 0.05$) different from diet A value. [4] Glutamic acid (50%) in diet A was replaced by glutamine.

Pair-feeding experiments. The results in ad libitum feeding experiments show that the growth depression of rats fed amino acid diet may be due to a reduction in food intake. If food intake is the only major factor influencing growth, amino acid diet should have provided the same growth as casein diet when casein diet was pair-fed with amino acid diet. The second group of bars in figure 2 shows a growth rate of 3.6 g/day with amino acid diet B versus 4.0 g/day with casein diet C, despite equalization of intake of diet B with diet C (10.0 g/day). Although the difference in growth rates of both the dietary groups was small ($P > 0.05$), the difference was observed in the repeated experiments. Therefore, certain factors other than food intake are presumed to influence the growth rate.

Space-feeding experiments. Since rats could not eat as much amino acid diet as casein diet under ad libitum conditions, the feeding of diets in a short period such as space-feeding may contribute to marked decrease in food intake of rats fed amino acid diet. To ascertain this assumption three kinds of space-feeding experiments were conducted. The results are summarized in figure 2. When rats were fed daily in a single 2-hour period, a severe reduction in the growth rate and food intake occurred in rats fed casein diet C and amino acid diet B. As might be expected, rats could not eat sufficient amounts of diet B required for growth in a short period, so that the difference in weight gain of rats fed diet B and diet C was more pronounced than under ad libitum conditions. With increasing frequency of meals, total daily food intake of both dietary groups increased. When diets were provided twice or thrice daily, rats had similar growth rates and consumed almost the same amounts of diet as those under ad libitum feeding conditions.

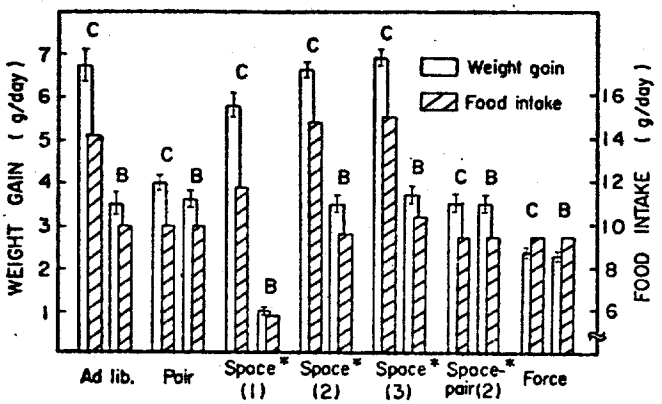

Fig. 2 Comparison of growth rate and food intake of rats fed casein diet C (C) with those of rats fed amino acid diet B (B) in several feeding methods.

As mentioned above, the most severe depression of food intake was observed when rats were space-fed amino acid diet once daily. This may be due to the osmotic effect of the amino acids as suggested by Rogers and Harper (21).

Space-pair-feeding experiments. The method of space-feeding (the ingestion of diet for 1 hour twice daily) was applied to two pair-fed partners in order to equalize not only food intake but also the number of meals and meal periods. Introduction of the space-pair-feeding method gave the same growth rate to rats fed amino acid diet B and casein diet C. The identity between both dietary groups was observed not only in the average growth rate (3.6 g/day) shown in figure 2, but also in the growth curve during the 14-day experimental period as shown in figure 3. The results of the experiments indicate that the amino acid mixture simulating casein was nutritionally equivalent to intact casein when each rat of the casein group was space-pair-fed with an animal fed amino acid diet.

Force-feeding experiments. By force-feeding, feeding conditions can be exactly equalized between rats fed casein and amino acid diets. As presented in the last group of bars in figure 2, amino acid diet B provided a growth rate equal to that obtained with casein diet C (2.4 g/day) when rats were force-fed the same amounts of diets (9.5 g/day). But rat growth in the tube feeding experiments was inferior to that in the space-pair-feeding experiments in spite of equal food intake.

DISCUSSION

In ad libitum feeding experiments, the effect of casein on rat growth was compared with that of the amino acid mixture simulating casein (fig. 1). At dietary nitrogen levels of 3.2 and 4.8%, rats of the casein group grew more rapidly and ate larger amounts of the diet than the amino acid group. Similar results were reported by Rogers and Harper (21) and by Adkins et al. (22), but they used a mixture with an amino acid pattern different from that of casein in the nutritional comparison with casein. Rogers and Harper (21) provided amino acid diet in agar gel form on the assumption that the diet in gel form might

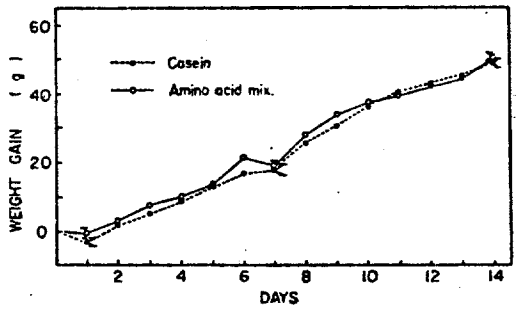

Fig. 3 Average weight gain of rats space-pair-fed casein diet C and amino acid diet B for 14 days. Each point represents the average of eight animals (the mean ± SEM on days 1, 7 and 14).

reduce osmotic effects in the gastrointestinal tract, and found an increase in intake of the agar gel diet. Harper and Spivey (23) found an inverse relationship between the capacity of a dietary carbohydrate to exert osmotic pressure and the food intake. These results of earlier investigations on food intake suggest the osmotic effect of dietary amino acids as a factor causing the lower intake of amino acid diet at higher nitrogen levels. Our results obtained at lower dietary nitrogen levels were in fair agreement with the earlier observations by Sauberlich (8) that growth of weanling rats fed an amino acid diet simulating 10% casein diet, approximated that of rats fed diet containing 10% of intact casein. In similar experiments at a dietary nitrogen level of 1.8%, however, Rama Rao et al. (12) observed differences in weight gain and food intake. Dietary energy may be an explanation of the disagreement in results: dietary gross energy was 3.5 kcal/g of diet in the study of Rama Rao and 4.1 kcal/g of diet in the present study.

Energy intake was shown to affect the magnitude of superiority of casein over amino acid mixtures by Rose et al. (24). Nitrogen balance of men fed intact casein was better than that of men fed acid-hydrolyzed casein at an energy intake of 35 kcal/kg body weight/day, while the men retained similar amounts of nitrogen from both sources at an intake of 45 kcal/kg body weight/day. A significant interaction was observed between energy level and nitrogen source in animal experiments. Ahrens et al. (14) pair-fed young rats intact casein and the amino acid mixture simulating casein. When energy intake was 34 kcal/day, there was no difference in nitrogen storage between the two groups; however, when energy intake was 48 kcal/day, casein was superior to amino acid mixture in nitrogen storage. The results at lower energy intake agreed with the growth study by Stucki and Harper (25) at an energy level of about 34 kcal/day, although they did not use an amino acid mixture simulating casein. In the present study energy intake in pair-feeding experiments was 41 kcal/day corresponding to the mean of two levels, 34 and 48 kcal/day. The results shown in figure 2 agree with those at the higher energy level rather than those at the lower level examined by Ahrens et al.

Data in our pair-feeding experiments (fig. 2) indicate the possible presence of certain factors influencing the growth rate other than food intake. When casein diet was pair-fed with amino acid diet on a daily basis, rats receiving casein diet were in a hungry state, because they were not provided enough food to satisfy the hunger. Thereby, rats of the casein group tended to consume their diet in a relatively short period, while rats of the amino acid group could eat their diet whenever they wanted. When the difference in feeding conditions was minimized by space-pair-feeding for 1 hour twice daily, the growth curves of rats of both groups were the same during the 14-day experimental period (fig. 3). The findings were also confirmed by the experiments force-feeding the same amounts of casein and amino acid diets. Thus, the difference in growth rate of amino acid and casein groups pair-fed on a daily basis might be due to the differences in number of meals and meal period. This assumption is supported by the report of Muiruri and Leveille (26) that rats of a space-fed group gained more weight than rats fed ad libitum in spite of equal food intake. From the results of the space-pair-feeding and the force-feeding experiments the amino acid mixture simulating casein was considered to be as effective as intact casein under the conditions of restricting the intake of casein diet to that of amino acid diet and equalizing feeding conditions in both the dietary groups.

To achieve our final purpose, that is, preparing a nutritionally superior amino acid diet, further comparative experiments must be performed under the conditions of equalizing intake of amino acid diet to that in ad libitum feeding of casein diet.

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Measurement of brain uptake of radiolabeled substances using a tritiated water internal standard

The method reported here permits the convenient regional measurement of uptake by animal brain of ^{14}C or ^{35}S labeled substances which enter brain tissue with some degree of freedom. The method uses [^{3}H]water as an internal reference standard of brain uptake.

A mixture of the labeled test substance and [^{3}H]water is injected into the rat common carotid artery with decapitation 15 sec later. The ratio of ^{14}C to ^{3}H in brain tissue relative to the ratio of ^{14}C to ^{3}H in the original mixture determines the amount of test substance lost to brain tissue on a single passage through brain microcirculation. The injected mixture distributes both to the external carotid distribution and to brain. Essentially all of the [^{3}H]water which enters brain is lost to brain tissue from blood and the amount of ^{3}H in the portion of brain examined defines the amount of injected mixture which distributed to that piece of tissue. A variable amount of the test substance will have left the microcirculation and entered brain as a function of blood–brain barrier (BBB) permeability and the tissue distribution space of the test substance.

By 15 sec after injection the test substance remaining in the blood is carried on out of brain circulation. This period of clearance is based upon studies using [^{14}C]-inulin mixed with [^{3}H]water assuming the inulin is non-diffusible in brain in the brief exposure to the microcirculation during the test interval. [^{14}C]Sucrose with decapitation at 15 sec was also studied as a non-diffusible test substance. [^{35}S]L-methionine was studied as an example of a diffusible test substance.

The carotid injection is made at a rate which is sufficiently high to minimize mixing with rat plasma thereby allowing an isolated exposure of the test substance to BBB carrier sites without competition from substances in free solution in blood plasma. This isolation of the injected solution allows the inclusion of various non-radioactive substances in the injected solution to assess their effects on the uptake of the radiolabeled test substances.

A mixture of the ^{14}C or ^{35}S labeled test substance and [^{3}H]water, containing approximately 1 μCi of each radionuclide in 0.2 ml Elliott's 'B' irrigating solution, is prepared in a tuberculin syringe.

Wistar rats of mixed sex, 300–350 g, on routine feeding, are rendered unresponsive with intraperitoneal pentobarbital. The rat is positioned supine on an operating board and the anterior neck skin incised and one of the common carotid arteries is exposed.

The carotid is punctured with a sharp 27 gauge needle and 0.2 ml of radioactive mixture injected during an interval of approximately 0.25 sec. The needle is left in place and 15 sec after injection the animal is decapitated. During this 15 sec interval it is essential that carotid flow past the puncture site be unimpeded so that any radionuclide not passing out of the microcirculation into brain tissue extracellular compartments will be carried on out of the brain vasculature.

The half of the brain rostral to midbrain and ipsilateral to the injection is quickly dissected free, placed in a 3 ml syringe and 0.2–0.25 g extruded through a 20 gauge

needle. The sample is subjected to routine digestion and preparation for liquid scintillation counting. Only enough brain tissue need be used to obtain statistically useful counts. An aliquot of the original isotope mixture is obtained by washing out the residual mixture in the syringe. Both this aliquot and the tissue sample are counted for ^{3}H and ^{14}C by routine liquid scintillation counting techniques. Neither the weight of injected solution or weight of brain tissue to be digested is recorded.

To determine the time of decapitation, the rate of clearance of a non-diffusible indicator was studied. Six groups of 3 rats were studied using [^{14}C]inulin with decapitation 2, 4, 6, 10, 14, and 18 sec after injection. Eight additional animals were studied using [^{14}C]sucrose, with decapitation at 15 sec.

To study the extraction of an amino acid, [^{35}S]L-methionine was used but [^{14}C]-methionine could have been substituted. The concentration of methionine in the injected solution was either 0.015 m*M* or 5 m*M*. The higher concentration was prepared by adding non-radioactive L-methionine to the labeled methionine. Three animals were studied at each concentration. An additional 12 animals were studied to determine the optimum volume of injection into the carotid artery. The study was conducted with 0.015 m*M* labeled methionine injected in volumes of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 ml.

The uptake by brain is calculated as follows where E, the extraction of the test substance relative to [^{3}H]water (100%), is:

$$E = \frac{^{14}C \text{ in brain tissue}/^{3}H \text{ in brain tissue}}{^{14}C \text{ in mixture}/^{3}H \text{ in mixture}} \times 100$$

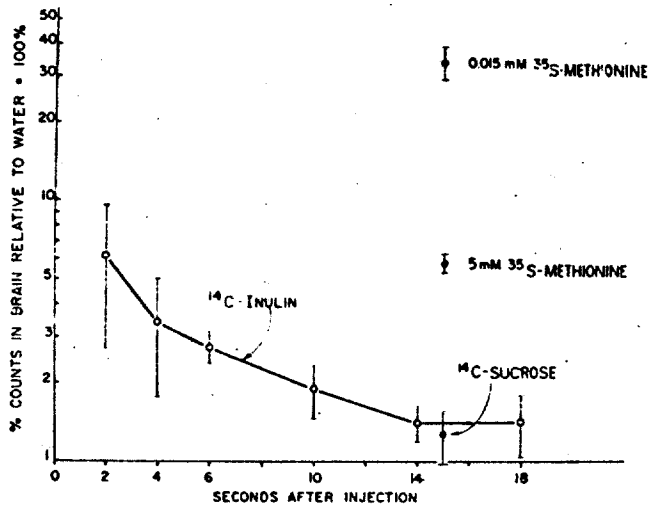

Fig. 1. The amount of test substance remaining in rat brain following carotid injection with decapitation at various times after injection. The residual inulin indicates the rate at which a non-diffusible substance is carried on out of brain. The inulin and sucrose persisting in brain tissue beyond 14 sec probably represent largely recirculation of the tracer. The difference in brain uptake of labeled methionine at low and high methionine concentrations probably represents saturation of a rate-limited passage of methionine through blood–brain barrier.

Fig. 1 indicates the percentages of test substances inulin, sucrose, and L-methionine remaining in brain relative to water (100%) at various times after carotid injection. Each point in Fig. 1 represents at least 3 animals. Inulin is essentially completely cleared by 15 sec. Remaining sucrose is 1.27% (± 0.30) at 15 sec. After 15 sec residual inulin and sucrose probably represent recirculation.

When 0.015 m*M* methionine was injected, 33.9% (± 5.4) of the methionine was lost to brain relative to [^{3}H]water. With 5 m*M* methionine only 5.79% (± 0.78) was lost to brain.

The studies conducted with 0.015 m*M* methionine using volumes between 0.05–0.5 ml showed no clearly definable difference in the extraction rate.

The tritiated water is assumed to come into nearly complete equilibrium with exchangeable water in brain during a single bolus passage and is, therefore, almost completely lost to brain tissue from brain blood after its passage following carotid injection[4].

It was considered of interest to determine the proportion of the common carotid injection which distributed to brain. Approximately 1 μCi of tritiated water in 0.2 ml of Elliott's 'B' solution was injected into the common carotid artery of 4 groups of 4 rats each decapitated at 1, 2, 8, and 15 sec after injection. An aliquot of injection solution was weighed and counted. The injection syringe was weighed before and after injection. The entire brain above the level of decapitation was removed, homogenized and an aliquot weighed and counted. The percentage of the injected solution present in brain was calculated. This percentage is expressed as a function of time in Table I. This indicates that approximately 8% of the injection distributes to brain and approximately one-third washes out by 15 sec. The remainder of the injected solution distributes to the external carotid arterial distribution and, by retrograde flow down the carotid, to other tissues.

TABLE I

PERCENTAGE OF TRITIATED WATER IN ENTIRE BRAIN OF THE RAT AS A FUNCTION OF TIME AFTER COMMON CAROTID INJECTION

Time of decapitation (sec)	*No. of animals*	*Percentage of ^3H_2O in brain and S.D.*
1	4	8.13 ± 1.58
2	4	7.16 ± 1.29
8	4	6.62 ± 1.15
15	4	4.97 ± 0.18

The curve for inulin indicates virtually complete disappearance from brain after 14 sec. Inulin is assumed confined to blood in brain, and the diminishing amount present for a few seconds after carotid injection represents prolonged transit paths through the brain blood compartment. Although decapitation time is not critical, 15 sec was selected on the basis of the sequentially timed inulin studies (Fig. 1). This time

of sacrifice should be as early as possible to minimize tissue washout of water and late enough to assume all of the non-extracted substance has passed out of the brain. Some of the test substance is always present at 15 sec due to recirculation.

The reduction of the methionine uptake by brain when in high concentration in the injected solution probably represents saturation of the BBB carrier system facilitating passage of methionine.

The volume of solution routinely injected in these studies is 0.2 ml. This was chosen because it seemed a convenient volume to inject into the rat common carotid artery through a needle small enough not to interfere with continued carotid circulation and because of the absence of a correlation between uptake and injected volume, as observed using 0.015 m*M* methionine in volumes up to 0.5 ml. During the brief period of injection the artery clears of blood and immediately after the injection, normal flow resumes. Thus the injected solution arrives in the brain microcirculation at approximately its original concentration in the syringe and undergoes minimal dilution by blood.

Because of the generally higher specific activity and lower cost of tritiated compounds, certain experimental situations might warrant the use of tritiated test substances. In this circumstance a ^{14}C labeled lipid-soluble, preferably non-volatile, diffusible substance, such as antipyrine, might be substituted for the tritiated water internal standard in the present technique.

The double-indicator method of Chinard *et al.*[1] is commonly employed to measure the extraction of a test substance by an organ during a single microcirculatory passage following arterial injection. A mixture of a non-diffusible substance (such as labeled serum albumin or Evan's Blue) and a test substance is injected as a bolus into the arterial supply of the organ. Serial venous samples are drawn during the period of efflux of the bolus from the organ. By knowing the ratio of non-diffusible reference substance to the test substance in the injected mixture, the percentage of test substance during organ passage can be calculated from measurements of the two labeled substances in the venous effluent. This method measures extraction during a single microcirculatory passage and, like the method described here, is limited to substances to which the organ capillary bed is appreciably permeable.

In brain the Chinard method has been extensively applied by Crone[2,3]. This method requires cannulation of the venous drainage of the organ under study. In most organs this is easily accomplished. The venous drainage of brain in small animals is difficult to isolate from other cranial tissues. The method does not allow measurement of regional differences since the venous effluent from the entire organ is mixed. These limitations cause the method to be poorly suited to brain measurements: especially in small animals.

The method presented here can be considered an inverse of the Chinard method, and is particularly suited to small animal brain studies. It substitutes tritiated water, a highly diffusible reference substance, for the non-diffusible reference substance. Calculation of extraction of the test substance is based on measurement of radioactivity in brain tissue rather than venous blood. It allows a more convenient measurement of regional BBB permeability to any radiolabeled substance which enters brain with some degree of freedom.

The restricted permeability of BBB limits the measurement of extraction to lipid-soluble substances or to substances entering brain by carrier-mediated transport. This, nevertheless, includes many drugs, glucose, amino acids, and perhaps other substances related to brain metabolism. The present method should allow a greater accuracy than the Chinard method in measurements of substances showing only a few percent extraction. Such substances must be measured with great accuracy in the venous effluent samples in the Chinard method since they differ from the non-diffusible reference by only a few percent. In the present method the extracted substance is determined directly rather than by the difference between two large, nearly identical values for reference and test substances.

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Uptake of Radiolabeled Essential Amino Acids by Brain Following Arterial Injection (35270)

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The requirement of brain for amino acids is met either by synthesis from other amino acids or glucose *in situ* or from the blood amino acid pool. Uptake of amino acids from blood requires passage through the blood–brain barrier (BBB) which is highly impermeable to most molecules the size of amino acids. The observed selective free passage of some amino acids from blood to brain is attributed to carrier mediated BBB transport (1).

We have studied the uptake of 18 labeled amino acids by rat brain after carotid arterial injection and find a great variability in the percentage of amino acid extracted during the first few seconds after injection. The data indicate amino acids essential for brain are taken up from blood to a greater degree than those which can be synthesized within brain from other substrates.

Method. A mixture of approximately 0.5 μCi each of ^{14}C-labeled amino acid and ^{3}H water in 0.2 ml of a physiological salt solution (Elliott's "B" solution, Baxter Lab, Morton Grove, Illinois) is injected (approx. 0.25 sec) into the surgically exposed common carotid artery of 300-g Wistar rats immobilized with pentobarbital, with decapitation 15 sec later. Injection is through a 0.4-mm diameter needle and is sufficiently rapid that the artery clears of blood during injection thereby minimizing mixing of the injected solution with blood. The cerebral hemisphere ipsilateral to the injection is quickly dissected free and subjected to routine liquid scintillation analysis for ^{14}C and ^{3}H. Some of the injected isotope mixture is similarly analyzed and the ratio of ^{14}C/^{3}H in brain is compared with the same ratio in the injected solution.

The percentage extraction (E) relative to water (100%) under these circumstances of injection is calculated by:

$$E = \frac{\text{brain tissue } ^{14}\text{C/brain tissue } ^{3}\text{H}}{\text{injected } ^{14}\text{C/injected } ^{3}\text{H}} \times 100.$$

Three rats were injected with each of 18 ^{14}C amino acids. ^{14}C mannitol was also studied (3 animals) as a metabolically inert substance with a molecular weight similar to the amino acids. The mannitol present in brain 15 sec after carotid injection probably represents both recirculation and residual tracer not yet washed out of the brain–blood compartment.

Results and Discussion. Approximately 8% of the injected solution distributes to brain, the remainder passing to the external carotid distribution (2). A large relatively fixed proportion of the tritiated water which enters brain is assumed still present at the time of decapitation, an equilibrium having been reached between brain exchangeable water and capillary water with virtually complete initial loss of labeled water from the capillaries during a single microcirculatory pass (3). A variable amount of the ^{14}C-labeled amino acid has left the capillary bed during the single microcirculatory pass and remained in the brain tissue. Approximately 40% of the initial brain uptake of ^{3}H water washes out during the first 15 sec (2). The labeled water remaining in the analyzed brain specimen serves as an internal standard against which to compare the amino acid extraction. By 15 sec, the amino acid not extracted is assumed to have been carried out of the brain circulation.

When arranged in descending order of extraction, Table I, there is no obvious correlation with molecular structure. However, 10 of the first 11 amino acids listed represent those considered nutritionally essential in the

TABLE 1. Percentage Uptake of ^{14}C L-Amino Acids and ^{14}C D-Mannitol Relative to $^{3}H_2O$ by Brain After Common Carotid Injection in Rat.

	Nutritional classification	(*E*) % Taken up by brain	
		Mean	SD ($n=3$)
H_2O		100	
Phenylalanine	Ess.	54.5	5.4
Leucine	Ess.	51.0	2.7
Tyrosine	Noness.	46.8	3.2
Isoleucine	Ess.	37.3	0.92
Methionine	Ess.	34.5	1.35
Tryptophan	Ess.	33.6	3.8
Histidine	Ess.	31.0	1.8
Arginine	Ess.	20.8	1.9
Valine	Ess.	19.8	2.2
Lysine	Ess.	13.9	2.5
Threonine	Ess.	10.7	0.23
Serine	Noness.	7.05	0.48
Alanine	Noness.	5.50	0.85
Citrulline	Noness.	4.71	1.19
Proline	Noness.	3.05	0.21
Glutamic	Noness.	2.81	0.15
Glycine	Noness.	2.47	0.25
Aspartic	Noness.	2.24	0.57
D-Mannitol	—	1.92	0.23

Relationship between classification of Rose *et al.* (4) into nutritionally essential or nonessential amino acids in rat and percentage taken up by brain after carotid injection. Amino acid concentration injected was different for each acid depending on the specific activity of the labeled material with a range of 0.003 to 0.05 m*M*. Radiochemicals were from New England Nuclear, Boston, or from Amersham/Searle, Arlington Heights, Illinois.

rat (4). The exception, tyrosine, considered nutritionally nonessential in the rat (4), is readily derived from phenylalanine by hepatic phenylalanine hydroxylase. The large influx of tyrosine noted here suggests tyrosine in rat brain is derived from blood and is compatible with the observed absence of phenylalanine hydroxylase in rat brain (5). These data suggest that although tyrosine is nonessential in rat extraneural tissues, it is essential in brain.

After intravenous injection of ^{14}C-labeled glucose, ^{14}C rapidly appears in brain alanine, aspartic, and glutamic acids and, to a lesser extent, in serine and glycine (6). This suggests some, at least, of the brain requirement for these amino acids is met from a glucose source.

The amount of test substance extracted by brain is a function of its brain tissue distribution space and BBB permeability. Entrapment and utilization by brain cells increase the brain distribution space and would be expected to increase the amount of test substance extracted. A high BBB permeability should increase brain uptake. The relative importance of these factors in determining the amount of a given substance extracted by brain remains unsettled.

Summary. ^{14}C-labeled amino acids were injected into rat common carotid artery mixed with $^{3}H_2O$, with decapitation 15 sec later. The ratio of ^{14}C to ^{3}H in brain was compared with the same ratio in the injected solution allowing expression of brain uptake of amino acid as a percentage of $^{3}H_2O$ uptake. Eighteen amino acids showed a range of uptake between 2.24 and 54.5%. Those amino acids ordinarily considered nutritionally essential in the rat are taken up to a greater extent than nonessentials. The exception is tyrosine which is not nutritionally essential to the total organism because it is available from hydroxylation of phenylalanine. The large uptake of tyrosine by brain may be related to the absence in brain of phenylalanine hydroxylase thus necessitating an external source.

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Monosodium Glutamate Metabolism in the Neonatal Pig: Effect of Load on Plasma, Brain, Muscle and Spinal Fluid Free Amino Acid Levels[1]

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ABSTRACT Fasted 3-day-old pigs were given by stomach tube 0.01, 0.10 and 1.0 g/kg body weight of monosodium glutamate dissolved either in water or infant formula. At appropriate times plasma, spinal fluid, muscle and brain samples were obtained for amino acid analysis. The levels of glutamate administered included those which could have been ingested by the human infant (0.01 and 0.10 g/kg) and a level ten times greater (1 g/kg). A marked difference in glutamate absorption was noted depending upon the presence or absence of food in the gut. Plasma glutamate levels were elevated maximally 20 minutes after glutamate was administered in water and 90 to 120 minutes after administration in infant formula. Maximum plasma concentrations were essentially the same with both methods of administration. No significant differences in plasma amino acid levels were noted between control animals and those given 0.01 g/kg body weight of glutamate. When glutamate was given at 0.10 g/kg body weight, small elevations in plasma glutamate and aspartate were noted at maximal absorption times. Marked elevations in concentrations of plasma glutamate, aspartate and alanine were noted following a dose of 1 g/kg body weight glutamate. In fasting animals portal plasma glutamate levels were five times greater than peripheral plasma levels. No significant changes in brain, muscle or spinal fluid free amino acid concentrations were noted in animals studied at the 0.01 and 0.10 g/kg body weight level. J. Nutr. *103*: 1138–1145, 1973.

INDEXING KEY WORDS monosodium glutamate · glutamate · amino acids

Glutamic acid is a common amino acid constituting about 20% of total amino acids found in natural protein sources. Monosodium glutamate (MSG) is a widely used food flavor enhancer whose use dates to antiquity. In 1957 Lucas and Newhouse (1) observed that suckling mice injected with MSG at 2.2 g/kg body weight daily for 14 days developed retinal lesions, a finding confirmed by other investigators in both mice and rats (2–6). Adult mice were more resistant to glutamate than the newborn animal, and Lucas and Newhouse noted that glutamate injection of pregnant mice produced no observable abnormalities in the offspring (1). Olney and his collaborators (7, 8) reported that the accurate nucleus of the hypothalamus was particularly vulnerable to MSG-induced lesions in the infant mouse, rat, rabbit and a single immature rhesus monkey injected subcutaneously with doses of MSG ranging from 0.5 to 2.7 g/kg body weight. Olney and Ho (9) also reported similar lesions in the infant mouse after oral ingestion of 3 g/kg body weight MSG, aspartate or cysteine. Following these reports, other research groups (10, 11) confirmed the presence of a hypothalamic lesion in the newborn mouse administered large quantities of MSG.

In species other than the mouse, the production of this lesion is a subject of considerable controversy. Arees and Mayer (12), Burde et al. (11) and Everly (13) have reported lesions in the rat. Adamo

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and Ratner (14) were unable to produce the rat lesion and Oser et al. (15) were unable to produce the lesion in either the rat or dog.

The nature and extent of the lesion reported in the MSG-treated primate has also been the subject of considerable controversy. The original publication of primate data by Olney and Sharpe (8) involved a single premature rhesus monkey which exhibited an extension lesion in the arcuate nucleus of the hypothalamic region following injection of MSG (2.7 g/kg body weight). The appearance and extent of the lesion was reported to be similar to that noted in the mouse. Subsequently, Reynolds et al. (10) and Abraham et al. (16) reported their failure to observe the lesions as reported by Olney and Sharpe (8), noting that inadequately fixed tissue has the same appearance as the published photomicrographs of the original monkey lesion (10). These reports apparently prompted Olney and Sharpe to perform additional experiments administering MSG orally (17). As a result of these studies, the lesion formerly designated as extensive and similar to the mouse lesion, is now reported to be extremely small in size (50 to 90 cells), seen only in thin epon sections, and found only upon careful scrutiny of serial sections of the arcuate area. Thus, at the present time it is at least safe to say that considerable difference in MSG response is noted between the rodent and the primate. Little is known about the significance of the "microlesion" which appears to involve approximately 50 to 90 cells. It will be interesting to see if other laboratories can confirm this microlesion. These findings, which differ on the basis of species and age of animal, make it imperative to acquire data on the rate of MSG uptake and metabolism in the mouse and more neurologically mature neonates such as the pig or monkey. Comparison of metabolic pathways for oral versus injected MSG must be made. In the latter case the gut and the liver, two major organs controlling plasma amino acid levels, are bypassed. In addition, the doses studied should include those which could possibly be ingested by the human infant. We have studied the effect of a MSG load on the plasma, brain, muscle and spinal fluid amino acid concentrations in the newborn pig. MSG was administered either in water or infant formula. The levels of MSG studied included those which might be fed a human infant ingesting its total daily calories from commercial infant foods containing the highest concentration of added MSG.[2]

MATERIALS AND METHODS

Three-day-old pigs were removed from the sow to heated cages and fasted for 6 hours. MSG, dissolved either in 5 ml of water or infant formula,[3] was administered by stomach tube at 0.01, 0.10 and 1.0 g/kg body weight. Control animals received either water or formula. Blood samples were obtained from the anterior vena cava, prior to administration of the MSG load and at the specified time intervals noted, by the method of Baker and Andresen (18). Two or four hours after administration of MSG the animal was anesthetized with chlorohydrate and samples of spinal fluid and portal blood were obtained. The animals were killed by exsanguination and samples of muscle (psoas) and brain (hypothalamic area) removed postmortem. Tissue samples were immediately frozen in liquid nitrogen and stored at −70°. In some experiments the jugular vein was catheterized to obtain sequential blood samples. Plasma was deproteinized with solid sulfosalicylic acid (19) and analyzed immediately or frozen at −70°. Spinal fluid samples were prepared by the method of Dickinson and Hamilton (20). Tissues were prepared for amino acid analysis by homogenizing with 10 volumes of 3.5% sulfosalicylic acid for 3 minutes in a Virtis homogenizer. The precipitated protein was removed by centrifugation at 20,000 × *g* for 15 minutes at 4°, and 1-ml aliquots of the supernatant solution were assayed. These techniques avoid conversion of glutamine to glutamate or pyrrolidone carboxylate (21–23). Amino acid determinations were carried out on NC-1 amino acid analyzers,[4] using the buffer system described by Efron (19). Monosodium glutamate[5]

[2] Since December 1969 commercial infant foods have not contained any added MSG.
[3] Similac Liquid Concentrate: Ross Laboratories, Columbus, Ohio.
[4] Technicon Instruments, Tarrytown, N. Y.
[5] International Minerals and Chemical Corp., Skokie, Ill.

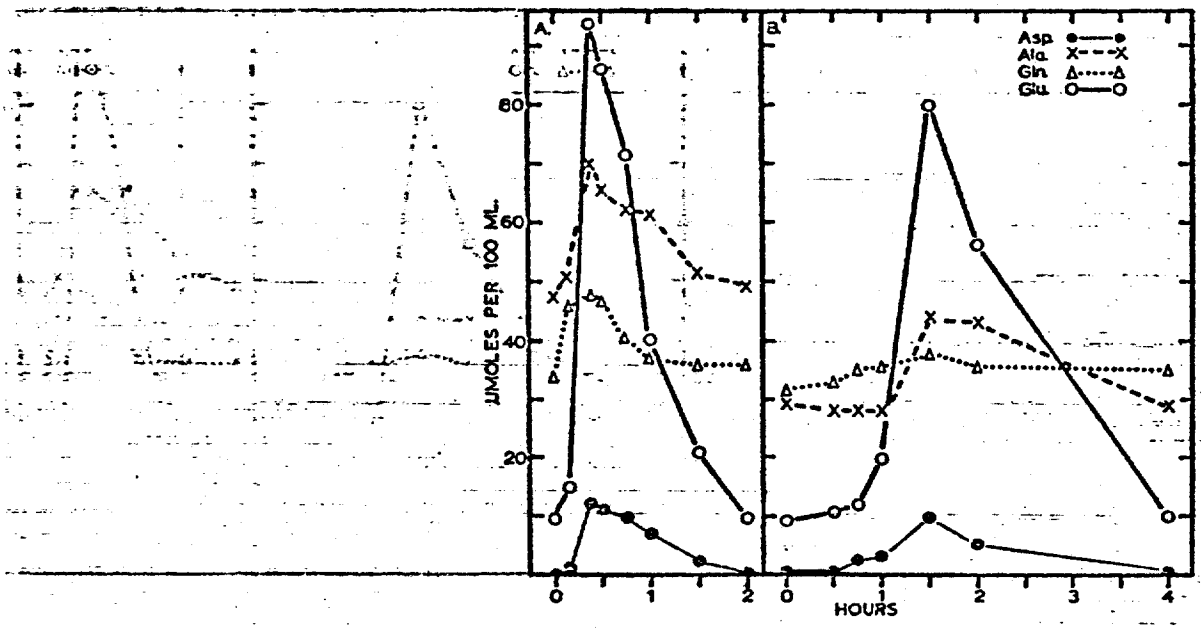

Fig. 1. Plasma amino acid levels in a typical neonatal pig following an oral load of 1 g/kg MSG administered in water (A) and in infant formula (B). Blood samples obtained from the vena cava.

assayed at greater than 99.9% MSG upon amino acid analysis.

RESULTS

Initial studies to determine the effect of food in the gastrointestinal tract on the rate of glutamate absorption were carried out following administration of a load of 1 g/kg body weight MSG in either water or infant formula. As shown in figure 1, a marked difference in glutamate and alanine absorption was noted depending upon the presence or absence of food in the gut. Plasma glutamate levels reached a maximum 15 to 30 minutes following administration of the load in water. When MSG was administered in infant formula, plasma glutamate levels were elevated maximally 90 to 120 minutes after the load. Maximum plasma concentrations were essentially the same in both feeding regimens. Changes in plasma aspartate, alanine and glutamine at the 1 g/kg load were significant, but less pronounced. Other amino acids showed no consistent pattern of change.

Changes occurring in plasma amino acid concentrations of young pigs administered lower doses of MSG in water are shown in table 1. No significant differences were noted between control animals and those given 0.01 g/kg body weight of MSG. At the 0.1 g/kg body weight load of MSG small elevations in plasma glutamate and aspartate levels were noted 15 to 30 minutes following administration. At this level only plasma aspartate and glutamate levels at 15 to 30 minutes differed statistically from those of control animals ($P \leqq 0.05$). Plasma glutamate and aspartate levels fell rapidly to normal by 60 minutes. The slight elevation in plasma glutamate concentration noted in control animals and those given 0.01 g/kg body weight MSG are in accord with the effect of stress on plasma glutamate concentration reported by Heath and co-workers (24). Animals given a 1.0 g/kg MSG load had major elevations in plasma glutamate levels, with lesser increases in plasma alanine, aspartate and glutamine.

The response of plasma amino acid levels to the administration of MSG in infant formula is shown in table 2. No differences were noted between control animals and those given 0.01 g/kg body weight MSG. Slight elevations in the plasma concentra-

TABLE 1

Plasma (vena caval) free amino acid levels in neonatal pigs given MSG with water

Group	N	Free amino acid levels					
		0 min	15 min	30 min	60 min	90 min	120 min
		μmoles per 100 ml					
Glutamate							
Control	6	9.4 ±2.4	14.8. ±4.3	13.1 ± 2.7	11.8 ±3.1	9.5 ±2.1	9.7 ±3.0
0.01 g/kg	6	9.2 ±2.0	15.1 ±3.9	12.7 ± 2.3	12.1 ±2.9	9.6 ±2.0	9.6 ±2.2
0.1 g/kg	5	9.0 ±2.8	21.2 ±5.1†,[1]	18.5 ± 3.2**	9.8 ±2.9	9.7 ±1.2	9.6 ±2.0
1 g/kg	3	9.1 ±2.1	45.7 ±8.8*	98.3 ±15.3*	41.3 ±7.9*	19.9 ±7.6*	9.7 ±3.4
Aspartate							
Control	6	1.01±0.57	1.08±0.41	1.09± 0.59	1.06±0.52	1.08±0.51	1.07±0.30
0.01 g/kg	6	1.05±0.62	1.08±0.56	1.08± 0.40	1.08±0.58	1.08±0.52	1.08±0.53
0.1 g/kg	5	1.09±0.59	2.01±0.55**	3.05± 0.49*	1.03±0.61	1.09±0.41	1.02±0.49
1.0 g/kg	3	1.02±0.48	8.41±1.75*	13.8 ± 3.98*	8.27±2.20*	2.31±0.69***	1.06±0.22
Glutamine							
Control	6	48.8 ±7.3	47.5 ±6.9	49.2 ± 9.0	47.9 ±8.5	49.7 ±8.6	48.7 ±6.9
0.01 g/kg	6	48.6 ±7.1	47.5 ±7.2	48.7 ± 8.3	47.7 ±6.9	49.0 ±7.1	49.1 ±6.8
0.10 g/kg	5	47.9 ±6.4	43.1 ±6.2	54.8 ± 7.6	50.8 ±6.7	48.7 ±6.9	48.0 ±7.1
1.0 g/kg	3	48.3 ±7.5	57.1 ±6.2	58.7 ± 7.1	48.6 ±8.1	47.7 ±6.3	49.1 ±8.3
Alanine							
Control	6	42.2 ±8.1	48.5 ±6.3	50.3 ± 6.8	45.8 ±7.5	44.7 ±6.1	46.1 ±7.2
0.01 g/kg	6	42.5 ±7.3	42.7 ±6.5	43.1 ± 6.0	41.9 ±7.2	44.1 ±6.2	45.7 ±6.8
0.10 g/kg	5	43.2 ±6.9	51.3 ±6.2	55.1 ± 7.3	50.3 ±6.9	44.7 ±7.5	44.2 ±8.1
1.0 g/kg	3	46.2 ±8.1	58.7 ±8.9	67.4 ± 9.0**	62.3 ±8.2***	50.1 ±7.4	48.2 ±8.9

[1] Differs significantly from control; * $P \leq 0.001$, ** $P \leq 0.02$, *** $P \leq 0.03$, † $P \leq 0.05$.

TABLE 2

Plasma (vena caval) free amino acid levels in neonatal pigs given MSG with infant formula

Group	N	Free amino acid levels			
		0 min	60 min	90 min	120 min
		μmoles per 100 ml			
Glutamate					
Control	6	9.6 ±2.7	9.7 ± 3.2	10.2 ± 2.9	9.9 ± 1.9
0.01 g/kg	5	9.7 ±3.0	8.9 ± 2.7	9.5 ± 3.1	9.4 ± 2.6
0.10 g/kg	6	9.8 ±3.1	11.8 ± 3.2	14.9 ± 3.4	12.8 ± 3.2
1.0 g/kg	3	9.6 ±2.8	31.2 ±10.1**,[1]	92.3 ±17.9*	58.3 ±14.8*
Aspartate					
Control	6	1.05±0.61	1.05± 0.32	1.11± 0.02	1.32± 0.45
0.01 g/kg	5	1.15±0.45	0.95± 0.33	1.05± 0.37	1.20± 0.54
0.10 g/kg	6	1.13±0.51	1.80± 0.60	2.80± 0.95**	1.60± 0.80
1.0 g/kg	3	0.97±0.43	4.11± 1.50**	10.5 ± 4.11*	5.01± 2.50***
Alanine					
Control	6	42.3 ±8.2	45.6 ± 6.4	48.3 ± 7.2	48.1 ± 6.5
0.01 g/kg	5	40.5 ±6.7	44.7 ± 6.2	48.2 ± 4.9	44.3 ± 5.8
0.10 g/kg	6	46.3 ±6.9	48.3 ± 5.8	54.3 ± 6.8	50.1 ± 6.8
1.0 g/kg	3	43.7 ±7.5	55.3 ± 7.8	68.3 ±12.1†	56.3 ± 7.2
Glutamine					
Control	6	48.3 ±7.1	46.3 ± 6.8	49.3 ± 9.1	47.3 ± 7.6
0.01 g/kg	5	48.3 ±6.7	48.7 ± 6.7	46.7 ± 8.2	49.0 ± 6.9
0.10 g/kg	6	47.6 ±6.4	47.2 ± 6.4	55.1 ± 8.9	52.3 ± 6.8
1.0 g/kg	3	46.3 ±7.8	46.3 ± 8.2	58.3 ±12.2	55.7 ± 7.6

[1] Differs significantly from control; * $P \leq 0.001$, ** $P \leq 0.002$, *** $P \leq 0.01$, † $P \leq 0.02$.

TABLE 3

Free amino acid concentrations in selected tissues of neonatal pigs given MSG

Tissue	Group	N	Amino acid				γ-Amino-butyrate
			Aspartate	Glutamine	Glutamate	Alanine	
			μmoles per gram of wet weight tissue				
Muscle	Control	6	0.87±0.22	3.35±0.97	2.91±1.04	4.02±0.94	0
	0.01 g/kg	5	0.70±0.07	3.93±0.75	2.20±0.44	3.32±0.74	0
	0.10 g/kg	12	0.95±0.15	4.15±0.70	2.90±0.89	4.59±0.77	0
Brain	Control	6	8.44±0.72	4.82±0.60	7.29±1.25	1.72±0.20	5.76±0.65
	0.01 g/kg	5	8.25±0.51	5.37±0.54	7.60±0.32	1.71±0.10	5.88±0.77
	0.10 g/kg	12	7.83±2.33	4.62±1.38	6.30±1.58	1.81±0.54	4.59±1.82

tion of glutamate and aspartate were noted 90 to 120 minutes after administration of 0.1 g/kg body weight MSG. In general, plasma glutamate levels were lower in animals given MSG in formula than in those where MSG was administered in water. The elevation observed, however, persisted for a longer period of time in the formula-fed pigs. Marked elevations in plasma glutamate, aspartate and alanine concentrations followed a load of 1 g/kg body weight MSG.

The lack of an effect of an MSG load upon free amino acid concentrations in muscle and brain of neonatal pigs is shown in table 3. Concentrations of γ-aminobutyrate, an important brain metabolite of glutamate, are also shown. The tissues of these animals were removed upon termination of the experiment which ranged from 30 to 120 minutes after the administration of the load. Because of the schedule of administration, no tissues were obtained from animals shortly after administration of MSG at 1 g/kg body weight. Tissues which were obtained 24 hours after loading showed no significant increase over control levels. Free amino acid concentrations in cerebrospinal fluid were unchanged (table 4).

From these data it is obvious that the liver converts a considerable quantity of ingested glutamate into a variety of other metabolites. This is particularly striking if the concentrations of various amino acids present in portal blood are considered. Portal and vena caval plasma free amino acid concentrations for control animals fasted 4 hours are compared in table 5. When compared to vena caval blood, plasma glutamate levels are markedly elevated in portal blood even in the absence of MSG loading. Thus, the liver removes a considerable quantity of glutamate from portal blood, converting it to other metabolites. The high concentration of glutamate in portal blood most likely arises from the large amount of endogenous protein secreted into the lumen of the gut (25).

DISCUSSION

There appears to be no doubt that large doses of intraperitoneally injected glutamate will cause specific lesions in the hypothalamic region of the newborn mouse (10–12). However, the effect of MSG on the central nervous system of newborn animals of other species is still controversial.

The question of possible toxicity of ingested glutamate to the human newborn

TABLE 4

Free amino acid concentrations in cerebrospinal fluid of neonatal pigs given MSG

Treatment	N	Amino acid		
		Glutamine	Glutamate	Alanine
		μmoles per 100 ml		
Control	6	55.5±11.7	1.81±0.51	9.69±3.0
0.01 g/kg	5	47.4± 6.0	2.32±0.61	10.7 ±5.5
0.10 g/kg	12	49.4± 5.38	2.61±1.85	8.63±1.5

must involve consideration of the route by which this amino acid enters the circulation. With oral alimentation, glutamate enters the gut and is carried by the portal circulation directly to the liver. The liver, one of the major organs controlling plasma amino acid concentrations, rapidly alters these levels to accommodate the needs of the organism. This is best illustrated by comparison of portal and peripheral blood glutamate concentrations in control animals. Under normal circumstances, glutamate is found at relatively low concentrations (5- to 10 μmoles/100 ml) in the peripheral blood of most species whereas considerable quantities of glutamine (50 to 70 μmoles/100 ml) are present. On the other hand, portal blood contains high levels of glutamate (48 μmoles/100 ml) even in the absence of a glutamate load. Thus a considerable quantity of ingested glutamate is converted into other metabolites by the liver.

The metabolic route of glutamate injected intraperitoneally differs from that entering via the gut. Since intraperitoneally injected glutamate does not pass through the liver prior to entering the circulation, a greater rise in plasma glutamate level is to be expected. Thus the production of hypothalamic lesions in the young animal injected with large loads of glutamate is not directly applicable to animals in which the glutamate is administered by the oral route.

Although we have measured the response of all plasma free amino acids to a glutamate load, data on only five amino acids are reported. This is based on our observation that substantial quantities of radioactivity are incorporated into only a few plasma amino acids following administration of U-^{14}C-MSG with unlabeled carrier at a level of 1 g/kg body weight (26). Most of the radioactivity is found in glutamate but detectable quantities are incorporated into glutamine, aspartate, ornithine, alanine, citrulline, and arginine. Of these, only aspartate, glutamate, and to a lesser degree, alanine and glutamine show a significant rise in absolute concentration with time, while no increase in concentration of the others is noted. We have included data on plasma and tissue concentrations of glutamine and γ-aminobutyrate since glutamate is a direct precursor of glutamine, and γ-aminobutyrate is an important metabolite of glutamate in the brain. Alanine concentrations are included because Wiseman and collaborators (27–29) reported that ingested glutamate appears in mesenteric blood as alanine in the dog and rabbit. Alanine may be formed by a glutamate-dependent transamination of pyruvate, with pyruvate being formed either from carbohydrate via glycolysis or from glutamate itself. In the pig, we have noted relatively small increases in plasma alanine concentrations following administration of large doses of MSG compared to the marked rise in plasma glutamate concentration during this same time interval. We have failed to detect any statistically significant increase in portal blood alanine levels following administration of low levels of glutamate (0.01 and 0.1 g/kg) although such changes may be masked by the large variability in portal alanine concentrations (table 5). Sequential sampling of portal blood from the same animal will be required to resolve this problem.

TABLE 5

Comparison of portal and vena caval plasma free amino acid concentrations. Control animals fasted 4 hours

Amino acid	Vena caval (N = 6)	Portal (N = 5)
	μmoles per 100 ml	
Aspartate	1.15±0.51	0.69± 0.32
Glutamine	48.4 ±7.35	41.2 ±14.3
Glutamate	9.61±3.27	48.3 ±12.4
Alanine	40.5 ±8.27	72.3 ±31.8

Dent and Schilling (30) have reported that portal blood concentrations of all amino acids, except glutamate, increase in proportion to their concentration in ingested casein. Later, Pion et al. (31) reported similar data for the adult pig fed various diets. Portal blood glutamate and aspartate levels were less than expected, while portal alanine and glycine concentrations were greater than expected, suggesting a glutamate-to-alanine conversion in the gut. However, it is not known whether the effect of glutamate on alanine concentrations involves the utilization of glutamate nitrogen to transaminate endogenous pyruvate derived from glucose, or whether

the carbon skeleton of glutamate itself is incorporated into alanine. We have suggested that for man the carbon skeleton of alanine is derived from sources other than glutamate (32). Such observations are consistent with data presented in the following paper (33) which demonstrate that little radioactivity from carbon-labeled glutamate is found in the peripheral plasma alanine of the neonatal pig. It is possible, however, that enzyme immaturity in the neonatal pig may limit conversion of glutamate to alanine. Since very large doses of MSG are required to produce appreciable increases in blood and tissue concentrations of glutamate, the pig obviously has considerable capacity to metabolize ingested MSG. Only when intake of this amino acid exceeds the capacity of the liver to metabolize glutamate are plasma levels substantially elevated. Changes in plasma amino acid levels of the newborn pig given 0.1 g MSG/kg body weight were similar to those noted in lactating women given a comparable dose (32).

Since little consideration has been given to the time sequence of glutamate absorption in other studies, it is difficult to interpret response to the ingestion of MSG (34, 35). In general, sampling times were considerably after peak elevations had occurred and no consideration was given to the effect of the presence of other amino acids and proteins on absorption. Our data are similar to those reported by McLaughlan et al. (36) who found no change in brain glutamate concentrations in adult rats given 2 g/kg MSG. Schwerin et al. (37) have reported that the intravenous injection of glutamate (1.2 g/kg body weight) into mice produced no change in brain glutamate concentration. It should be pointed out, however, that the time of sampling after glutamate injection and the particular area of the brain analyzed are of importance. In a paper which appeared after this study had been completed, Perez and Olney (38) measured glutamate levels in hand-dissected sections of infant mouse brain after a 2 g/kg glutamate subcutaneous load. Their data indicate an increase in glutamate levels of the arcuate nucleus 1 to 3 hours after injection. Glutamate levels of the ventromedial hypothalamus and lateral thalamus were not affected. Significant elevations in brain glutamate concentration were not noted in the total hypothalamic region assayed in our study. These samples were obtained 90 to 150 minutes after an oral load. It should be noted, however, that a maximum blood glutamate concentration of 4,000 μmoles per 100 ml was attained in the subcutaneously injected mice studied by Perez and Olney (38), and levels of 100 μmoles/100 ml were noted 3 hours after injection. This elevation is approximately 1000 times greater than normal levels, 200 times greater than the levels we note in the pig given an oral load of 0.1 g/kg body weight and 40 times greater than the levels noted in animals receiving an oral dose of 1 g/kg body weight. These observations emphasize the importance of considering the route of administration in studies of potential glutamate toxicity.

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Monosodium Glutamate Metabolism in the Neonatal Pig: Conversion of Administered Glutamate into Other Metabolites in vivo[1]

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ABSTRACT Recent studies of monosodium glutamate toxicity indicate a species specificity between mouse, pig and monkey. It is not known whether glutamate or one of its metabolites is responsible for the observed neurotoxic effects. U-^{14}C-glutamate, dissolved in either water or infant formula, was administered to newborn pigs at a level of 1 g/kg body weight. Blood samples were collected sequentially with time, and the incorporation of label into both plasma and amino acids and other metabolites was measured with time. Administration of the labeled glutamate resulted in the rapid labeling of plasma glutamate, arginine, aspartate, glutamine, alanine, ornithine, citrulline, urea and two nonamino acid metabolites. These metabolites were isolated and identified as glucose and lactate. The bulk of the radioactivity (65 to 80%) was found in glucose, glutamate and lactate at all time periods studied. Radioactive glutamate was more rapidly removed from plasma than glucose or lactate. No substantial radioactivity was noted in plasma succinate, pyrrolidone carboxylate, malate, citrate or oxaloacetate, although very small quantities of label were noted in pyruvate and α-ketoglutarate. Comparison of the plasma radioactivity profile at the point of maximal labeling with the cerebrospinal fluid radioactivity profile at this same end-point demonstrated that neither labeled glutamate nor aspartate entered the spinal fluid despite elevated plasma levels. Substantial quantities of label were noted in glutamine, glucose, lactate and urea of spinal fluid and plasma demonstrating rapid equilibration of these compounds between the two compartments. J. Nutr. *103*: 1146–1154, 1973.

INDEXING KEY WORDS MSG · glutamate · amino acids

When monosodium glutamate (MSG) is injected or fed to certain species at levels ranging from 0.5 to 3.9 g/kg body weight, an extensive lesion is observed in the hypothalamic region of the brain. Species specificity is suggested by these observations. The mouse (1–4) and possibly the rat (4–8) appear susceptible to extensive MSG-induced neuronal damage; however, studies in the dog and monkey have generated considerable controversy. In 1969, Olney and Sharpe (9) reported an extensive MSG-induced neuronal lesion in a premature primate. Other investigators, however, failed to reproduce this lesion as originally described (3, 10). In 1972 Olney et al. (11) redescribed in the primate the site and size of the original lesion following oral administration of large doses of MSG. The lesion formerly designated as extensive and similar to the mouse lesion was now reported to involve as few as 50 to 90 cells seen only by the electron microscope in ultra-thin serial sections (1 μ or less) of the hypothalamus. At the present time the significance of this "microlesion" in the primate is unknown and awaits further study. However, the marked difference in size and type of lesion observed between the neurologically immature neonatal mouse and the more neurologically mature

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neonatal pig or monkey is striking (1–3, 10–12).

The failure of several investigators (3, 10) to detect an extensive neuronal lesion in the newborn monkey administered large oral doses of MSG (1 to 4 g/kg body weight) has given rise to the proposition that a metabolite of glutamate is responsible for neuronal damage in the newborn mouse.

The toxic compound appears capable of penetrating the neural system of susceptible species during the early days of life but is unable to do so upon maturation of the animal (1). In an attempt to determine what differences if any are found in glutamate metabolism between the mouse and other species we have determined the major metabolites of glutamate released by the liver to the circulation which might affect the brain. In these studies we administered U-^{14}C-MSG to the newborn pig by stomach tube and measured the incorporation of glutamate into both amino acids and other metabolites in the peripheral circulation. Major nonamino acid metabolites have been identified.

MATERIALS AND METHODS

The L-monosodium glutamate used [2] assayed at greater than 99.9% glutamate by amino acid analysis. No other amino acids were noted. The L-U-^{14}C-MSG [3] used had a specific activity of 200 mCi per millimole, and was diluted with unlabeled MSG as noted.

Monosodium glutamate dissolved in water was administered by stomach tube to 3-day-old pigs which had been fasted for 5 to 6 hours prior to administration of the load. Sequential blood samples were obtained from each animal by means of a catheter placed in the external jugular vein. Cerebrospinal fluid samples were obtained by ventricular tap. Simultaneous radioactivity and amino acid analysis was carried out on each sample by the method of Stegink (13). This technique permits detection of both ninhydrin-negative and ninhydrin-positive metabolites derived from glutamate. Replicate analyses of physiological fluid samples yielded a variability of 10% or less.

For identification of the ninhydrin-negative metabolites of glutamate, pigs were given large loads of glutamate (1 g/kg body weight). Blood samples were obtained at the previously determined peak of radioactivity for the required compound.

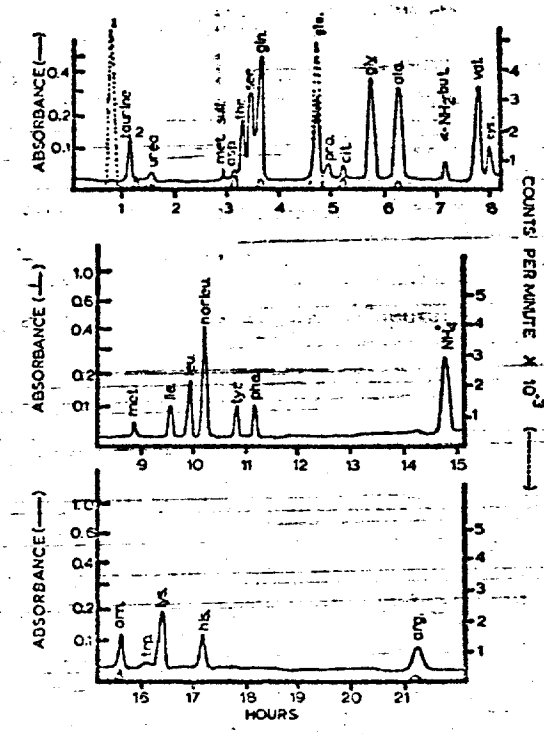

Fig. 1 A typical simultaneous radioactivity–amino acid analysis elution profile of plasma obtained 60 minutes after administration of MSG in water using the specially modified amino acid analyzer described (13). The abscissa lists the elution time from the analyzer column in hours. The ordinate lists both the absorbance of the eluate at 570 nm following reaction with ninhydrin (solid line) and the radioactivity detected (dotted line). The minor radioactivity peaks are drawn larger than actual size in order to demonstrate the definite presence of label at those positions. The precise radioactivity data are shown in figures 2 and 3.

The ninhydrin-negative compounds were isolated from plasma which had been immediately deproteinized either with sulfosalicylic acid (14) or by means of a Diaflo ultrafiltration cell. Samples of deproteinized plasma were applied to an amino acid analyzer column and the eluate collected in 2.0-ml fractions. The pattern of radioactivity obtained in this manner was identi-

[2] International Minerals and Chemical Corporation, Skokie, Ill.
[3] International Chemical and Nuclear Corporation, Waltham, Mass.

cal to that obtained using the simultaneous radioactivity amino acid analysis technique. Fractional collections were desalted by high voltage electrophoresis (15). This method gives excellent separation of acidic metabolites.

RESULTS

Administration of U-^{14}C-monosodium glutamate with cold carrier (10 μCi U-^{14}C-MSG and 1 g/kg body weight of MSG) to the newborn pig resulted in significant incorporation of label into glutamine, glutamate, aspartate, alanine, citrulline, ornithine, arginine, urea, and two major unidentified ninhydrin-negative compounds. A typical simultaneous amino acid–radioactivity analysis is shown in figure 1. The data in figures 2 and 3 demonstrate the rate of plasma metabolite labeling observed following administration of labeled glutamate in water (fig. 2) or infant formula [4] (fig. 3). The peak time of labeling of glutamate and aspartate corresponds to the maximal chemical elevation of glutamate and aspartate levels (16). Most of the radioactivity is found in glutamate and the unidentified ninhydrin-negative compound labeled "1." Smaller quantities of label are found in aspartate and ornithine. With increasing time after loading most of the radioactivity is found in the ninhydrin-negative compounds labeled "1" and "2," with much smaller quantities found in the amino acids. Of the various amino acids into which label was incorporated, only the absolute levels of glutamate, aspartate, glutamine and alanine were affected. No change was noted in the levels of other amino acids. The increase in plasma alanine concentration was more than could be accounted for on the basis of incorporated label, especially when the labeled MSG was administered in infant formula. These data are in agreement with our ob-

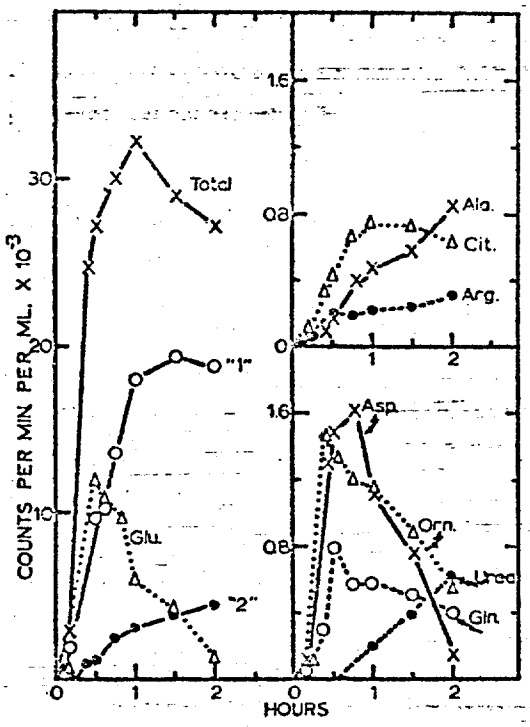

Fig. 2 Mean labeling rate of plasma metabolites following administration of a 1 g/kg load of MSC and 10 μCi of U-^{14}C-MSG in water to three neonatal pigs. Variability about each point does not exceed 15%.

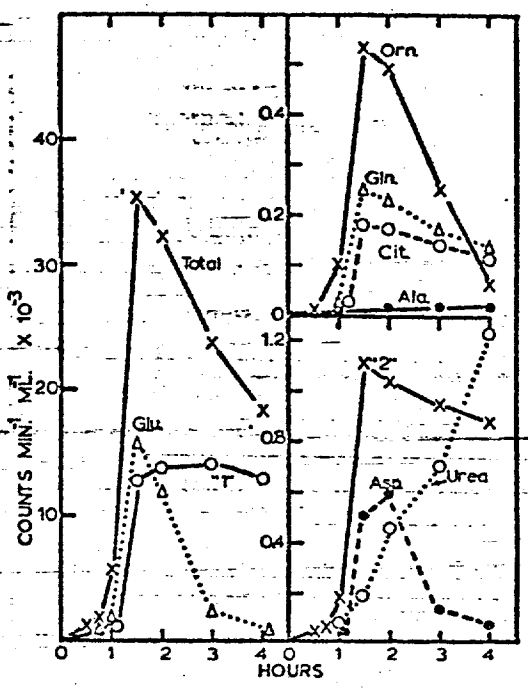

Fig. 3 Mean labeling rate of plasma metabolites following administration of a 1 g/kg load of MSC and 10 μCi of U-^{14}C-MSG in infant formula to three neonatal pigs. Variability about each point does not exceed 15%.

[4] Similac Liquid Concentrate: Ross Laboratories, Columbus, Ohio.

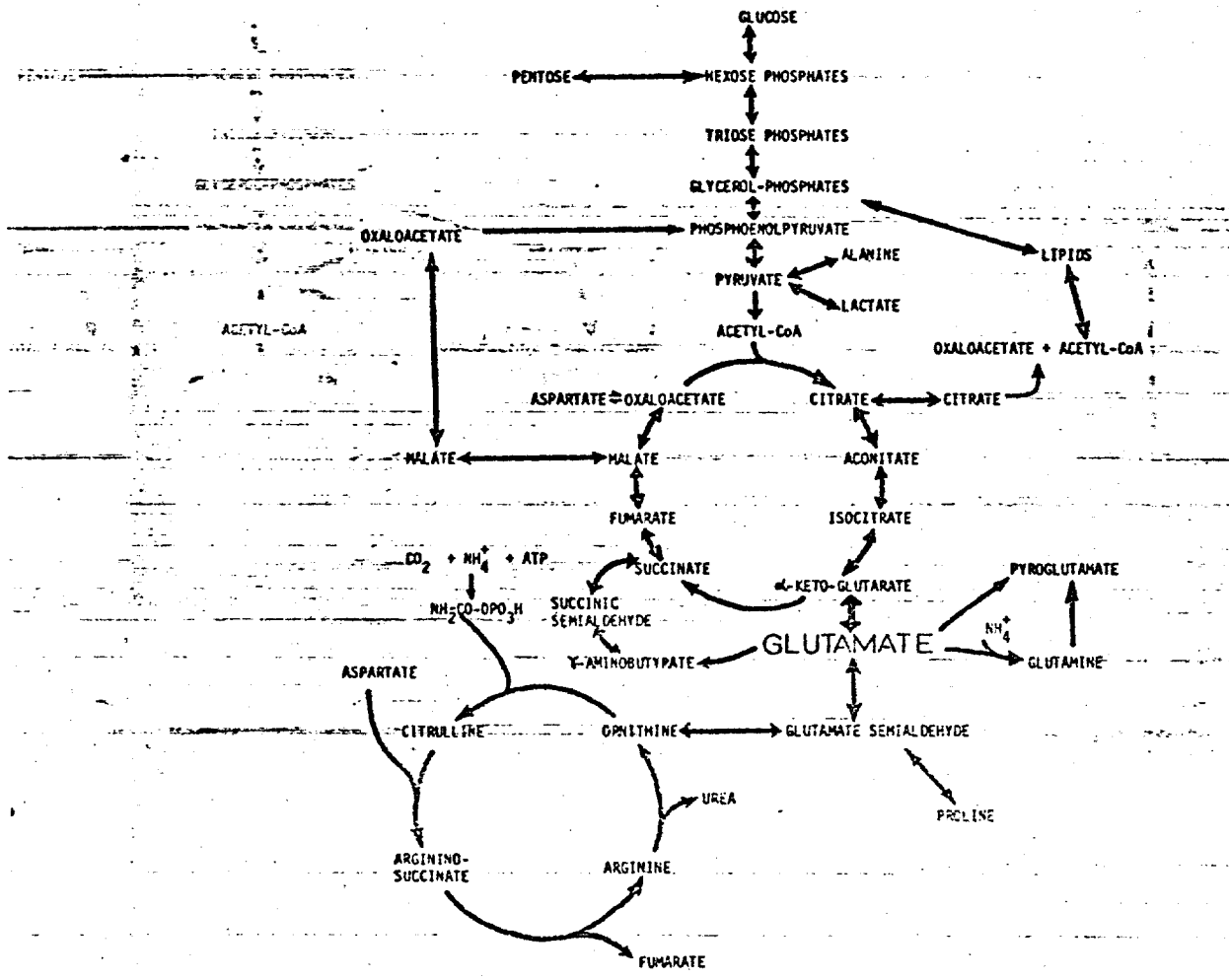

Fig. 4. Available pathways of glutamate metabolism.

servations in the human which suggest that glutamate provides little of the carbon structure of plasma alanine after a glutamate load (17).

Since it is not known whether glutamate or one of its metabolites is responsible for the neurotoxic effects observed in susceptible species, it was necessary to identify the ninhydrin negative compounds. The metabolic pathways open to glutamate indicate that a wide variety of compounds are possible (fig. 4).

Compound 1 was separated from other radioactive compounds using an amino acid analyzer column (13). Upon high voltage electrophoresis of this compound (4.5 kV 30 minutes, pH 3) only one radioactive peak was observed. This peak remained close to the origin as would a neutral compound. This method readily separates out most of the acidic metabolites which could be expected to be formed from glutamate, including those of the citric acid cycle. The radioactive spot did not react to the xylose–aniline–phosphoric acid spray for organic acids (18) or the Rydon-Smith chlorination spray (19) but did react with the aniline–diphenylamine reagent, the periodate–benzidine reagent, and the aniline–acetic acid–phosphoric acid reagent for sugars (20). Since glutamate is a gluconeogenic amino acid, it seemed likely that compound "1" was glucose. Compound "1" comigrated with authentic glucose in a

variety of paper chromatographic systems including butanol–acetic acid–water (4:1:1) and butanol–pyridine–water (4:4:1). The isolated compound had an NMR spectrum similar to that of glucose. The identification was completed by the reaction of compound "1" with glucose oxidase to yield gluconic acid (21). After reaction with glucose oxidase, the radioactive compound now had an R_F identical with gluconic acid and comigrated with gluconic acid in isopropanol–pyridine–acetic acid–water (8:8:1:4) solvent system. All of the radioactivity was found in either the residual glucose spot or in the gluconic acid spot.

Compound "2" was isolated in a similar manner from the amino acid analyzer effluent. Upon high voltage electrophoresis the compound migrated as a single spot which reacted with an organic acid spray but failed to react with general sugar sprays. Its position on the electrophoretogram corresponded to authentic lactate. It was well separated from the other potential acidic compounds that might arise from glutamate. The compound comigrated with authentic lactate when subjected to paper chromatography in ethanol–ammonium hydroxide–water (80:5:15). Identification was completed by conversion of the compound to pyruvate using the lactate dehydrogenase acetylpyridine-NAD^+ method (22). After treatment with lactate dehydrogenase and acetylpyridine-NAD^+, the compound migrated with authentic pyruvate in an electrophoretic assay.

The radioactivity of other compounds that are potential metabolites of glutamate were also measured. No substantial radioactivity was found in succinate, pyrrolidone carboxylate, malate, citrate or oxaloacetate following electrophoresis of deproteinized plasma. Whenever large amounts of plasma were applied to the electrophoretogram, very small quantities of radioactivity were noted in the pyruvate and α-ketoglutarate positions. We were able to confirm that α-ketoglutarate and pyruvate were labeled to a small extent by treating the deproteinized plasma with 2,4-dinitrophenylhydrazine to convert all keto acids to their dinitrophenylated derivatives. The dinitrophenyl derivatives were extracted and chromatographed as described by Smith and Smith (23). The majority (70%) of the radioactivity in this fraction was found in the dinitrophenyl–pyruvate spot while smaller quantities were noted in the dinitrophenylated-derivative of α-ketoglutarate. Using authentic standards we were able to demonstrate that α-ketoglutarate, oxaloacetate and pyruvate were eluted together from the analyzer column prior to the compound identified as glucose. Careful examination of the radioactive profile obtained from the simultaneous amino acid analysis technique revealed very small quantities of radioactivity present as pyruvate in our amino acid analyzer patterns. However, the maximum quantity of label noted was never more than 100 counts per milliliter, far below that noted in glucose and lactate.

Particular care was taken to eliminate pyrrolidone carboxylate as an important metabolite of glutamate, since a mentally retarded child exhibiting pyrrolidone carboxylate aciduria has been reported (24, 25). We were unable to detect any radioactivity at the pyrrolidone carboxylate position on either the amino acid analyzer profile or on the high voltage electrophoretogram. If pyroglutamate had been present, it should have been converted into glutamate by acid hydrolysis. A sample of deproteinized plasma which had been passed through a Dowex-50 column to remove free amino acids was subjected to acid hydrolysis and the hydrolysate analyzed using the simultaneous amino acid radioactivity technique. No radioactivity was noted in the glutamate position, demonstrating the absence of pyrrolidone carboxylate.

The importance of the simultaneous analysis technique in elucidating the cause of the lesion in susceptible species is demonstrated by the data in table 1. A 3-day-old pig was administered a load of MSG in infant formula [5] (1 g/kg body weight, 10 μCi U-^{14}C-MSG) by stomach tube. At the end of 2 hours, samples of peripheral blood and spinal fluid were obtained and subjected to simultaneous analysis for radioactivity and amino acid content. A comparison of the plasma radioactivity–amino acid pattern with that found in the spinal fluid of the animal at the same time (table 1) demonstrates that neither labeled gluta-

[5] See footnote 4.

mate nor aspartate entered the spinal fluid, despite elevated plasma levels both radioactively and chemically. Of the labeled amino acids only glutamine appeared at approximately the same concentration in both plasma and spinal fluid. Substantial quantities of radioactive glucose and lactate were present in spinal fluid and plasma, reflecting the rapid equilibration between compartments of these compounds in contrast to amino acids. Similar results were obtained in three other animals studied at 60, 90 and 180 minutes following administration of MSG.

TABLE 1

Radioactivity profile in physiological fluids of a neonatal pig loaded with 10 μCi ^{14}C-monosodium glutamate

Compound	Plasma	Spinal fluid
	cpm/ml	
Aspartate	640	ND*
Glutamine	220	130
Glutamate	13,573	30
Alanine	100	30
Ornithine	550	ND
Arginine	33	ND
Citrulline	180	ND
Glucose	14,200	11,460
Lactate	990	630
Urea	420	360

* ND = not detected.

DISCUSSION

Study of amino acid metabolism in man and experimental animals in vivo is hampered by the high degree of normal biological variation in physiological fluid levels and by the complex homeostatic mechanisms which control these levels. The use of a radioactively labeled amino acid allows measurement of the net flux of a specific labeled carbon atom but does not differentiate between the specific compounds involved in its metabolism. In addition, most amino acids are rapidly converted into ninhydrin-negative compounds which are no longer measurable by amino acid analyzer techniques.

Wainer (26) has pointed out that many ninhydrin-negative compounds are readily separated on the amino acid analyzer using conventional buffer systems and may be quantitated if a suitable method of detection such as radioactivity is available. The simultaneous measurement of radioactivity and amino acid composition which we have developed (13) and utilized in this study has proven invaluable in that it enables us to distinguish between the incorporation of label from an ingested labeled amino acid into other amino acids or ninhydrin-negative metabolites.

The major ninhydrin-negative metabolites isolated in this study do not appear to contain other metabolites as judged by high voltage electrophoresis and paper chromatographic techniques.

Conversion of ingested glutamate into lactate and glucose by the liver is reasonable. Portal blood normally contains high levels of glutamate even in the absence of glutamate loading when compared to peripheral blood. This observation indicates that the liver normally converts glutamate into other metabolites. Indeed the labeling of metabolites is logical when one examines the available metabolic pathways (fig. 4). Ingested glutamate is rapidly removed from the portal blood by the liver [see table 5 of (16)]. Once inside the liver cell, glutamate enters the mitochondria where it is rapidly converted into α-ketoglutarate and other tricarboxylic acid cycle components, principally malate and oxaloacetate. Oxaloacetate remains within the mitochondria, while malate is able to diffuse out. Mitochondrial oxaloacetate may be transaminated to aspartate which can be transferred to peripheral blood resulting in labeled aspartate. Cytoplasmic malate is converted into phosphoenolpyruvate. Phosphoenolpyruvate may be metabolized in a variety of ways depending upon the energy or oxidation–reduction status of the liver cell and its precise hormonal balance. In these studies, most of the phosphoenolpyruvate is converted into glucose, while smaller quantities passed through pyruvate into lactate. Since the mitochondria have sufficient quantities of α-ketoglutarate available for ATP synthesis, it is reasonable that the conversion of pyruvate into acetyl-CoA for oxidation in the mitochondria is decreased, with the majority of phosphoenolpyruvate being converted into glucose, along with smaller quantities converted into lactate.

Similarly, it is obvious that a portion of the α-ketoglutarate formed is oxidized to

CO_2 which may be incorporated into carbamylphosphate and ultimately find its way into urea. The incorporation of glutamate into ornithine, citrulline and arginine occurs to some degree indicating minimal conversion of glutamate into glutamate semialdehyde and subsequently to ornithine.

Conversion of glutamate into alanine does not appear to occur at a rapid rate. In view of the utilization of phosphoenolpyruvate for glucose synthesis, there is little indication that alanine formation would be favored by the liver. Our data indicate the presence of only trace quantities of α-ketoglutarate and pyruvate in the plasma after the 1 g/kg load. No detectable radioactivity was noted in plasma citrate, oxaloacetate, succinate, fumarate or malate by either high voltage or simultaneous amino acid analyzer techniques.

Previous experiments by Wiseman and his colleagues (27–29) indicate that in the dog and rabbit ingested glutamate appears in mesenteric blood as alanine. The adult pig has been reported to carry out a similar conversion (30). Our data indicate that this conversion may occur to a lesser extent in the neonatal pig. While we do not have portal blood samples at appropriate time intervals following loading, it appears that more of the ingested glutamate is found as portal blood glutamate than as alanine. In any case, only a small portion of the ^{14}C label is found as alanine in jugular vein plasma following administration of glutamate with water. When glutamate is given in conjunction with formula a somewhat larger quantity of the label appears as alanine. It is possible that alanine may carry the label in portal blood to the liver where it is rapidly converted into glucose and lactate. Experiments are presently in progress to differentiate this point. It may be that there is something unique about the absorption of glutamate as suggested by the earlier work of other investigators (27–31). Dent and Schilling (31) have reported that in the dog, portal blood concentrations of all amino acids increased in proportion to their concentration in ingested casein, except for glutamate which showed only a slight increase in concentration despite its high content in casein. Similar data were reported later by Pion et al. (30) in the pig. These latter authors noted lower postprandial portal blood glutamate and aspartate levels and greater portal alanine and glycine levels than expected, suggesting a glutamate to alanine conversion in the gut. Christensen and colleagues (32, 33) have shown that only a minor part of protein digested appears in the portal blood in peptide or conjugate form. However, these authors also noted that such conjugates were not necessarily fragments of the protein fed since feeding of L-glutamic acid also produced an increase in peptide conjugates in portal blood. It must be pointed out, however, that the absorption of peptides resulting from digestion of ingested intact protein in the gut is likely different from that of added individual amino acids. Sufficient data now exist (34, 35) to indicate that peptides are absorbed from the gut more rapidly than are their component amino acids, and that peptide metabolism in the gut mucosal cell may give rise to differing metabolic products than will the individual amino acids composing that peptide.

It is also to be expected that some of the ingested glutamate will be converted to acetyl-CoA and lipid components. In view of the general inability of thiol ester derivatives to pass through cellular membranes, it seems highly unlikely that acetyl-CoA would be released from the liver to the circulation.

Our data have relevance to data presented by Creasey and Malawista (36) suggesting that the toxic effect of MSG in the mouse results from the inhibition of glucose uptake in the brain. Their data indicate a decreased uptake of labeled glucose following pretreatment with glutamate, which they interpret as inhibition of glucose uptake. However, since a major quantity of ingested glutamate is converted into glucose in peripheral blood, simple dilution of the glucose pool could account for their results.

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